A COMPARATIVE STUDY OF MICROSCLEROTIAL DEVELOPMENT IN WILD TYPE AND MICROSCLEROTIAL MUTANTS OF VERTICILLIUM DAHLIAE

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ABSTRACT

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A Comparative Study of Microsclerotial Development in Wild-Type and Microsclerotial Mutants of Verticillium dahliae

Advisor: Dr. John E. Mayfield

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The purpose of this study was to compare a wild-type strain of Verticillium dahliae (t9) with two of its mutants, albino 4 (alm 4) and brown 1 (brm 1). Techniques of phase contrast microscopy and electron microscopy were used. The strains were grown in a medium which allowed synchronous development of microsclerotia and were harvested at 3 days. Microsclerotia resulted from conidia that had enlarged followed by germination. An analysis of conidia production suggested that their modes of microsclerotia production were basically the same. The most noticeable differences at the electron microscopic level were differences in nuclear structure and occurrence of single and double membrane-bound structures among the three strains. For example, alm 4 exhibited intensely stained chromatin material along with the appearance of virus-like particles. The single membrane-bound structures were found only in alm 4 and melanin-producing (t9) strains, while the double membrane-bound structures were found in all three strains.

ACKNOWLEDGMENTS

The author expresses great appreciation to Dr. John E. Mayfield for his guidance and assistance during this research. Appreciation also goes out to Dr. Enola Stevenson who served as a thesis committee member. A whole lot of thanks is extended to Mr. and Mrs. Anderson Gaskins for their ever-enduring parenthood.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Each year several million dollars are lost in damages to agricultural food crops. About 70% of this loss is caused by fungi. Fungi are also the causative agents for many human diseases such as athlete foot and many skin rashes. Fungi are believed to have appeared during the early evolution of the earth. Their evolutionary development has led to a wide variety of habitats and morphological forms. Despite the diversity among fungi, most have simple nutritional requirements consisting of mineral salts, carbon, and nitrogen sources. These simple requirements allow fungi to exist as either saprophytic or parasitic types.

Verticillium dahliae is a member of the form-class Fungi Imperfecti. The fungi in this form-class are characterized by the absence of an observed sexual phase in their life cycle (Wolfe and Wolfe, 1947, as cited in Harris, 1976). Previous studies on V. dahliae produced information on its pathogenicity, genetics, physiology, and cytology. Although there have been many attempts to define its life cycle, the actual life cycle is still relatively unknown.

V. dahliae reproduces by producing unicellular conidia which germinate to develop a filamentous hyphal form. Elongation of the hypha, as in other filamentous fungi, occurs at the

tip, thus extending the length of the hypha. As older hyphal regions are established, the cells become specialized. These specialized structures which are called microsclerotia (MS) are considered to be both survival structures (Schrieber and Green, 1962) and the sites for genetic variability (Tolmsoff, 1972) in V. dahliae.

Both survival and genetic variation play vital roles in the pathogenicity of V. dahliae. Since V. dahliae is a soil-borne pathogen, the fungus must survive the adverse conditions of the soil. Genetic variability is another means of assurring survival of the species. It seems that if Verticillium wilt is to be controlled, the MS stage of the life cycle is an excellent place to focus one's attention.

Prior to 1967 studies on the structure of MS had been confined to light microscopic techniques (Gordee and Porter, 1961; Schnathorst, 1965; Isaac, 1967) except for electron microscopic (EM) observations of Nadakavukaren (1963). More recent EM studies have involved MS from cultures (Brown and Wyllie, 1969) or host vascular tissue (Wright and Abrahamson, 1978). Since these reports Dr. W. J. Tolmsoff (1978) has developed a medium containing polygalacturonate, which promotes the synchronous development of MS. To date the only reported ultra-structural studies on cellular changes associated with MS production in synchronous cultures were conducted in this laboratory (Taylor, 1978).

Recently a series of mutants which vary in their ability

to produce normal melanin-containing MS were isolated (Bell et al., 1976). Such mutants are ideal for studying the process of melanin production and the association between melanin production and MS formation in V. dahliae. To date there have been no reported ultrastructure studies of the cytoplasmic organization of these mutants.

This study was undertaken to compare and contrast the wild-type (melanin producing) strain of V. dahliae with some of the mutants exhibiting alterations in melanin synthesis. The techniques of electron microscopy and phase contrast microscopy were employed to analyze microsclerotia development at the cellular level.

CHAPTER II

REVIEW OF LITERATURE

Verticillium dahliae, an imperfect fungus that produces unicellular conidia as its primary mode of reproduction, forms thick-walled melanized microsclerotia (MS) cells, which function as survival structures. The genus *Verticillium* was established in 1816 by Nees von Esenbeck (Schnathorst, 1973). *Verticillium* was first isolated from potatoes in 1879 by Reinke and Berthold who named it *V. albo-atrum*. In 1913, *V. dahliae* was isolated from diseased dahlia plants. In the years that followed there has been much controversy among mycologist and plant pathologist as to whether the two are the same or different species.

For example, Klebahn's publication in 1913 (as cited by Schnathorst, 1973) caused great controversy when he named a fungus from diseased dahliae plants *Verticillium dahliae*. He concluded that *V. dahliae* was different from Reinke and Berthold's *V. albo-atrum* and deserved species rank. He also suggested that the principal difference between the two fungi was the abundant production of MS by *V. dahliae* and their absence in cultures of *V. albo-atrum*.

Presley (1941) did not accept specific rank for these two fungi on the basis of some of the other reported works as well as his studies of variants derived from single spores of the MS form. He suggested that most of the forms in the

genus were merely variants of V. albo-atrum and should be designated as such. He also felt that if it is accepted that V. albo-atrum forms dark mycelial colonies and that V. dahliae forms abundant MS, both of these types were obtainable from a monosporic culture of the microsclerotial form.

Isaac (1949) working with a dark mycelial (DM) isolate suggested that it was the same one that Reinke and Berthold described. He also presented evidence that three distinct and constant fungal identities can be distinguished by morphologic, physiologic, and pathogenic criteria. He further concluded that whatever rank is given to the microsclerotial and dark mycelial forms, the differences remain constant and they are two distinct organisms or groups of organisms. He proposed that the name V. albo-atrum be used to designate the dark mycelial species and that V. dahliae be used for the microsclerotial form.

There have been many other studies done in an attempt to resolve this problem (Puhalla, 1973; Fordyce and Green, 1963). The overall results of these studies suggest that the most meaningful criteria for separating Verticillium spp. are morphology and certain physiological traits outlined by Isaac (1949, 1953), and that V. albo-atrum and V. dahliae are the valid, respective names of the (DM) and (MS) forms.

Current investigators tend to divert their attention

away from speciation and concentrate more on things like the life cycle, morphology and the metabolic processes such as melanogenesis. As an example, the life cycle of V. dahliae has received a great deal of attention. In spite of this attention its true life cycle has not been elucidated. An understanding of the life cycle of V. dahliae has met with many failures due to the number of technical problems associated with its relatively small cell size and dense hyphal growth pattern. These problems have greatly impeded research efforts and produced controversial conclusions with light and phase contrast microscopy. Despite the improved resolution of the electron microscope, there have been relatively few ultrastructural studies of V. dahliae.

V. dahliae normally grows as a mycelial form on agar plates, extending the colony's diameter by a process of elongation at the hyphal tip (Mayfield, et al., 1977). Progressing from the hyphal tip into older hyphal regions, there is a decrease in the length of the hyphal cells. Fusions between adjacent hyphae are observed about 1-2 mm behind the colony front (Puhalla and Mayfield, 1974). These hyphal fusions seem to serve as a mechanism of heterokaryosis between hyphae which contain nuclei of genetically different origins and possibly in the production of MS (Tolmsoff, 1972; Tolmsoff et al., 1973). Occurring about 2-5 mm from the colony front is a region of frequent conidiation. Conidia are produced in rapid succession on verticillate

arranged conidiophores (Mayfield et al., 1977). Beyond this region, the hyphal cells become shortened and exhibit an increase in diameter. These cells are MS initials which give rise to the MS located just behind this region.

The asexually produced conidia are considered to be the only recognized reproductive structure in the life cycle of V. dahliae. On the other hand, it has been reported that MS are the only recognized survival structure of V. dahliae. Microsclerotia are thick-walled melanized cells (Smith, 1965) that contain large lipid bodies and other cytoplasmic organelles (Tolmsoff et al., 1973; Brown and Wyllie, 1970). These large globose budding structures are characteristic of older hyphal regions in V. dahliae where they are believed to function as soil survival structures (Schrieber and Green, 1962) and as possible sites for genetic alterations (Tolmsoff, 1972). These genetic alterations must allow for the large number of variances occurring in V. dahliae cultures (Tolmsoff, 1972).

Heterokaryosis and parasexualism have been proposed as mechanisms of variations in fungi not having a sexual phase in their life cycle (Parmeter et al., 1963). The hypothesis of this dual phenomenon for V. albo-atrum was introduced by Hansen (1938). Using biochemical and morphological markers, Fordyce and Green (1963) were able to recover single spore colonies showing recombinant traits. These studies suggest that anastomosis and parasexual recombination

occurred between strains.

It has been suggested by Tolmsoff et al. (1973) that Verticillium must go through a haploid and diploid state before its life cycle can be completed. They further stated that when haploids are grown under conditions that prevent diploidization MS are not formed. Likewise, when diploids are grown under conditions that prevent haploidization, MS are not formed. The involvement of a parasexual cycle in V. dahliae was demonstrated by Puhalla and Mayfield (1974). They used heterokaryons to describe the mechanism by which the parasexual cycle might proceed. They were able to see the fusion of nuclei in heterokaryotic hyphae. These results tend to support Tolmsoff's hypothesis.

Microsclerotia of V. dahliae have received more attention at the ultrastructural level than any other stage of the obscure life cycle. This attention is well deserved since there is not sufficient subcellular data to definitely characterize the function of MS in V. dahliae. Nadakavukaren (1963) reported that there were heavy-walled cells containing mitochondria and other cytoplasmic organelles, and thin walled cells which contained only structures resembling nuclei or looked empty. He felt that only the thin-walled cells were capable of germinating. Brown and Wyllie (1970) later suggested that it was the thick walled cells that had intact cytoplasmic organization. Other investigators (Griffiths, 1970; Wright and Abrahamson, 1970) suggested that

there was really no difference in the thickness of the hyaline or pigmented cells, but the apparent difference was due to the deposition of material on the outside of the cell wall. This material is melanin and is associated with MS production. It has been suggested that melanin may be responsible for the survival of MS, since it has been shown to inactivate cell degradation enzymes (β-1,3 glucanase and chitinase) secreted by some soil organisms (Frederick and Newcomb, 1969; Bull, 1970). Melanin has also been implicated in protecting fungal cells from damage by irradiation and desiccation (Zhdanova and Pokhodenko, 1973).

Cultures of Verticillium grown in darkness are black with MS while those grown in near-uv are quite white and devoid of MS (Brandt, 1964). On the contrary, one may induce melanin to form in spite of the near u.v. by administering catechol after growth has proceeded for several days (Brandt, 1965). This treatment also induces MS to form. Therefore, it seems that a study of the enzyme system initiating melanin synthesis from catechol might help elucidate the basis for development of MS (MacMillan and Brandt, 1966).

The biosynthetic mechanism of melanin synthesis in the fungi is poorly understood. Several molecules such as 3,4 dihydroxyphenylalanine (DOPA) (Bull, 1970); 1,8-dihydroxynapthalene (Alport and Bulock, 1960); or catechol (Piattelli et al., 1965) have all been suggested as the substrate for melanin synthesis in fungi. Bell et al. (1976) have recently

isolated a series of mutants which exhibit different deficiencies in melanin biosynthesis. One of these mutants (brm), which produces brown MS instead of the usual black, accumulates a substance which enables an albino MS mutant (alm) to produce MS with the characteristic black pigmentation. The substance has been isolated and characterized as (+)-scytalone (3,4-dihydro-3, 6, 8-trihydroxy-1(2H) naphtalenone) (Bell et al., 1976). The purified compound can be converted to melanin by albino mutants of V. dahliae as well as by albino mutants of several other fungal species (Bell et al., 1976).

Although scanning and transmission electron microscopy studies have been aimed at understanding the deposition of melanin granules at the cell wall (Wheeler et al., 1976) and the critical morphology of various melanin granules (Wheeler et al., 1978), there has been only little work done on the cellular changes that accompany melanin synthesis.

Mayfield and Taylor (1978) did ultrastructural observations of cells at 2, 3, 4, 5, 10 and 20 days after inoculation. They were unable to find any significant changes in ultrastructure after five days, except for thickening of the cell wall. So, their ultrastructural results were from 2-4 day old cultures, with the most emphasis placed on the third day.

As reported by Mayfield and Taylor (1978), the 3 day old cultures were characterized by the presence of vacuoles

and small lipid granules. As development continued the lipid granules increased in size and number while mitochondria exhibited alterations such as the absence of cristae in portions of the mitochondria. By using 3,3-diaminobenzidine, cytochrome oxidase activity was demonstrable only in mitochondrial regions with intact cristae. The disappearance of cristae in the mitochondria coincides with the appearance of single membrane-bound granular vesicles which appear similar to the peroxisomes described in other fungi (Todd and Vigil, 1972) and plant material (Frederick and Newcomb, 1969). The vesicles have not been reported in any other structure (hyphae or conidia) of V. dahliae. Observations suggest that the mitochondria are converted to the peroxisome-like vesicles or play some role in their function. Therefore, altered mitochondria could be an intermediate structure for vesicles and peroxisome-like structures. While the changes occurring in the nucleus and mitochondria of the wild-type have been studied (Mayfield et al., 1977), there are no reported studies of the melanin-deficient mutants. Neither has there been a critical study of the functional state of the mitochondria as they are apparently converted to membrane-bound granular structures (Taylor, 1978).

Therefore, the plan of study entailed a critical ultrastructural study of the wild type (t9) of V. dahliae and two of its mutants, brown 1 and albino 4. Along with this, the

phase contrast microscope was used to study cellular changes and differences in the wild-type and the above two mutants at the light microscopic level.

CHAPTER III

MATERIALS AND METHODS

The strains of V. dahliae used in this study were the wild type (t9) and two mutants derived from it. The mutants were brm 1 and alm 4. All strains were obtained from Dr. Puhalla of the National Cotton Pathology Research Laboratory, College Station, Texas. Stock cultures were maintained on potato dextrose agar (PDA), prepared by the rehydration of 39 grams of dehydrated PDA (Difco) in 1000 ml distilled water. The medium was autoclaved for 20 minutes at 15 psi. It was then poured into disposable polystyrene petri dishes while still hot to reduce contamination. Upon solidification, they were inoculated under sterile conditions with sections from the peripheral regions of the colony.

Polygalacturonic acid medium (PGAM) was used for microsclerotia (MS) production (Wheeler et al., 1976). PGAM contained steam-warmed sodium polygalacturonate solution, 40 ml; agar (Difco), 2 g; KH_2PO_4, 52 mg; K_2HPO_4, 65 mg; $MgSO_4 \cdot 7H_2O$, 20 mg; yeast extract (Difco), 120 mg; peptone (Difco), 200 mg; and glucose, 700 mg; per liter. PGAM was dispensed in 50 ml quantities into 250 ml erlenmeyer flasks and autoclaved at 15 psi for 30 min. The PGAM was inoculated with hyphal tip regions from selected regions of the PDA plates. Once inoculated, the flasks were placed on a Lab-Line Orbit Environ

Shaker 18 and agitated constantly at 150 rpm at room temperature (24-28°C).

Phase Contrast Microscopy

Wet mounts of conidia and MS initials were prepared by placing a drop of PGAM culture suspension onto a clean microscopic slide with a sterile pasteur pipette. The microscopic samples were prepared and viewed 3 days after inoculation. All wet mount preparations were observed through a wild phase contrast microscope equipped for photography. Determinations of the conidial concentration were made by using a blood counting chamber. Conidial counts were made at 24 hr intervals from day 1 through day 5.

Since conidia develop into MS (Taylor, 1978), conidial suspensions of t9 were used to inoculate some PGAM cultures to determine the most suitable quantity of conidia for the best synchrony of MS production. Flasks of PGAM were inoculated with different quantities of t9. The amounts used were 1×10^3, 1×10^4, 1×10^5, and 1×10^6. These were allowed to incubate on the shaker at room temperature (24-28°C) for 5 days.

Transmission Electron Microscopy

Hyphae and MS to be observed with the electron microscope were harvested at 3 days from either PGAM cultures or PDA plates overlaid with cellophane. Using the PGAM cultures,

MS and intermediate stages were poured into centrifuge tubes and centrifuged for 15 min. Afterwards the supernatant was discarded and the remaining cells were then washed 3 times, each followed by centrifugation and discarding of the supernatant. Each step outlined below was followed by centrifugation and discarding of supernatant before proceeding to the next step.

Using a procedure described by Dr. H. H. Mollenhauer (personal communication), MS were placed in 3% glutaraldehyde, in 0.05 M sodium cacodylate buffer (pH 7.4) for 10-15 min at room temperature, then fixed for an additional 10-15 min in ice. MS were post-fixed using cold 2% OsO_4 in 0.05 M cacodylate buffer (pH 7.4) for 2 hr. The cells were then washed for 1 hr in cold distilled water with at least 10 changes and stained in 0.5% uranyl acetate for 30 min - 24 hr. The MS were washed in 3 changes of distilled water prior to dehydration. They were dehydrated in a graded series of ethanol to absolute acetone. The graded series were 50% ethanol; 70% ethanol; 95% ethanol; and 100% acetone. The cells were then infiltrated for 30 min - 24 hr in a mixture of acetone-Spurr's medium (1:1), followed by embedding in Spurr's low viscosity resin (1969). The resin was polymerized in Beem capsules at 65°C for 24 hr. Thin sections were cut on a LKB Bromma-Ultramicrotome with a diamond or glass knife, and collected on uncoated 300 mesh copper grids. Sections were post-stained with Reynold's (1963) lead citrate.

Sections were viewed with RCA EMU4 electron microscope operating with an accelerating voltage of 50 or 100 KV.

For the PDA plates over-laid with cellophane the selected regions of the plates were chosen and cut. Then, they were placed in vials containing 3% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) for 10-15 min at room temperature, then fixed for an additional 10-15 min in ice. This process follows basically the same steps as the one used for the PGAM cultures with a few differences. The differences are that no centrifugation is needed, since the tissues are in small mats. The solutions are just drained off with a sterile pipette after each step. Since these tissues are flat, they are flat-embedded. This process entails cutting the round ends off Beem capsules and closing the lids. They are turned upside down and the tissues matted against the flat end. This allowed the tissues to be flattened out. Also, tissues were embedded between two slides, which were later cut out and glued to the end of blank capsules with epoxy resin glue. The best results were obtained with a diamond knife. The blanks were made by filling the empty capsules with only Spurr's low viscosity resin and letting it polymerize. The slides were precoated with sigmacote (Sigma Chemical) to allow the tisuses embedded between them to be removed easily.

CHAPTER IV

RESULTS

Phase Contrast Microscopy

The flasks of PGAM inoculated by means of conidial suspensions contained 50 ml of PGAM initially. They were inoculated with conidial concentrations of 1 x 10^5 conidia/ml. Therefore, the initial conidial concentration of each flask was 2 x 10^3 conidia/ml of medium. After 24 hr of incubation alm 4 had a concentration of 3.0 x 10^5conidia/ml whereas brm 1 and t9 showed conidial concentrations of 4.5 x 10^5 and 5.5 x 10^5 conidia/ml, respectively (Table 1). After 48 hr the concentrations were 6.0 x 10^5, 1.6 x 10^6, and 1.15 x 10^6 conidia/ml for alm 4, brm 1, and t9, respectively. After 72 hr the concentrations were 9.0 x 10^5, 1.9 x 10^6 and 1.25 x 10^6 conidia/ml for alm 4, brm 1, and t9, respectively. When the final determinations were made at 96 hr alm 4, brm 1 and t9 had concentrations of 1.2 x 10^6, 24. x 10^6, and 2.1 x 10^6 conidia/ml, respectively. Although there were variations in the number of conidia in the medium, the relative multiplication patterns did not vary between the strains (Fig. 1).

There was a rapid increase in the number of conidia during the first 24 hr of incubation in t9 and both mutants

(alm 4 and brm 1). The variations in conidial concentration did not seem significant enough to suggest completely different developmental modes. Yet, these data show that there was a slight difference in the conidiation of alm 4 as compared to brm 1 and t9. As shown in Table 1, there was a constant rise in alm 4 from day 1-4, whereas brm 1 and t9 showed only a gradual increase from day 2-4.

Microsclerotia development of all strains was observed at the phase contrast microscopic level. It was determined that their modes of development were very similar. Therefore, micrographs of t9 were used to describe MS development. The first indication of MS development in PGAM was conidial enlargement (Fig. 2). Following enlargement the conidial issued germination (germ) tubes (Fig. 3). Small, enlarged and germinating conidia can be seen in Fig. 4. After emergence of the germ tubes, some began to develop conidiophores as they elongated (Fig. 5). This type of development preceded the appearance of young MS which occurred around the third day (Fig. 6).

Since it was obvious that conidia in PGAM gave rise to MS, erlenmeyer flasks were inoculated with different concentrations of conidia in order to determine the concentration for the best synchronous development of MS. Erlenmeyer flasks containing 50 ml of PGAM were inoculated with the following concentrations: 1×10^3, 1×10^4, 1×10^5, and 1×10^6 conidia / ml. They were labeled A, B, C, and D, respectively.

Table 1. Conidia production in alm 4, brm 1 and t9 from day 1-4 in PGAM.

Days following inoculation	Number of conidia/ml of medium		
	alm 4	brm 1	t9
1	3.0×10^5	4.5×10^5	5.5×10^5
2	6.0×10^5	1.6×10^6	1.15×10^6
3	9.0×10^5	1.9×10^6	1.25×10^6
4	1.2×10^6	2.4×10^6	2.1×10^6

Fig. 1. Conidial production by alm 4 ---○ , brm 1 ___□ , and t9 ___● in PGAM.

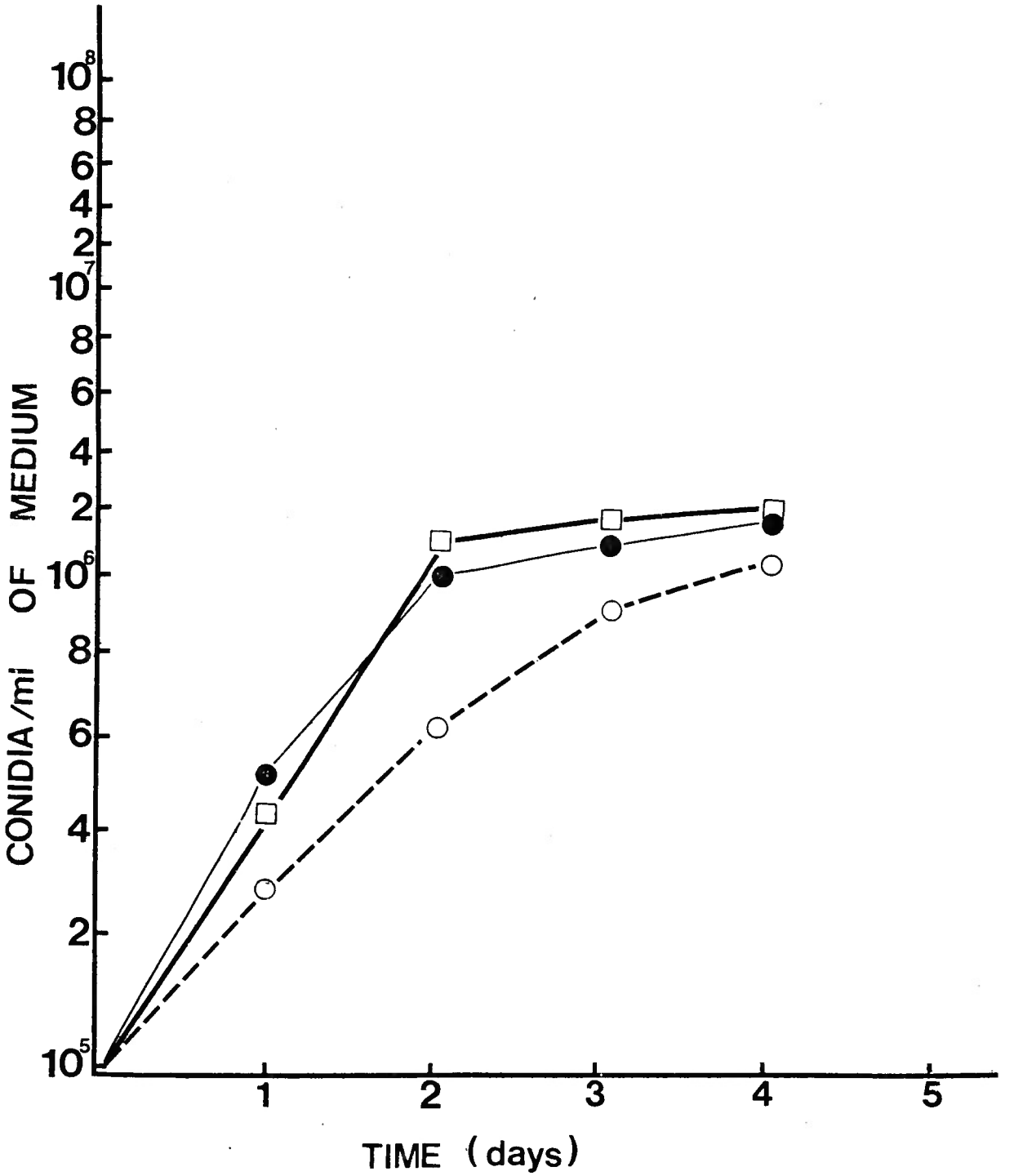
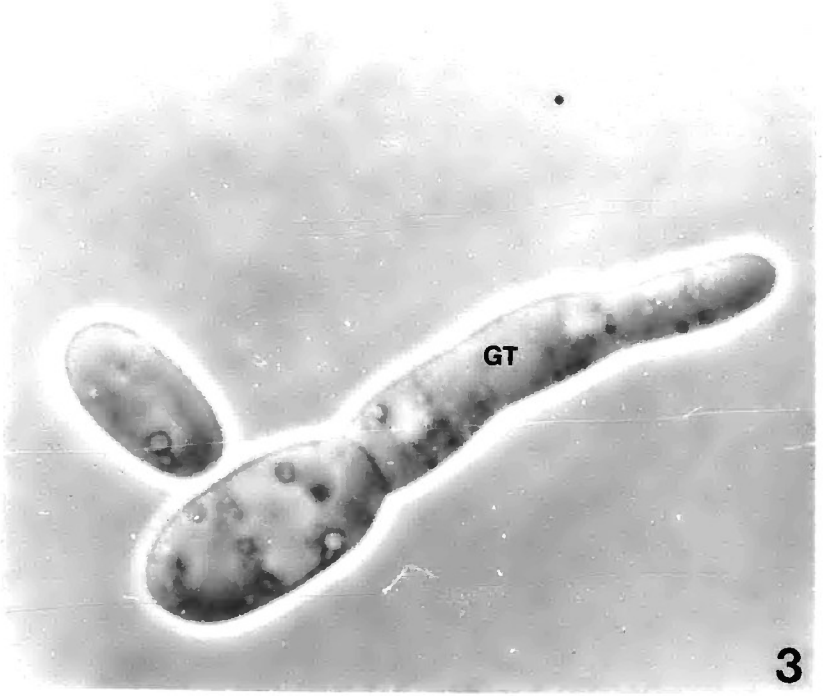

CONIDIA/ml OF MEDIUM
10^8
8
6
4
2
10^7
8
6
4
2
10^6
8
6
4
2
10^5
1
2
3
4
5
TIME (days)

Fig. 2. Enlarged conidia, the first sign of MS development in PGAM. X6,160.

Fig. 3. Enlarged conidium issuing a germ tube (GT). X6,160.

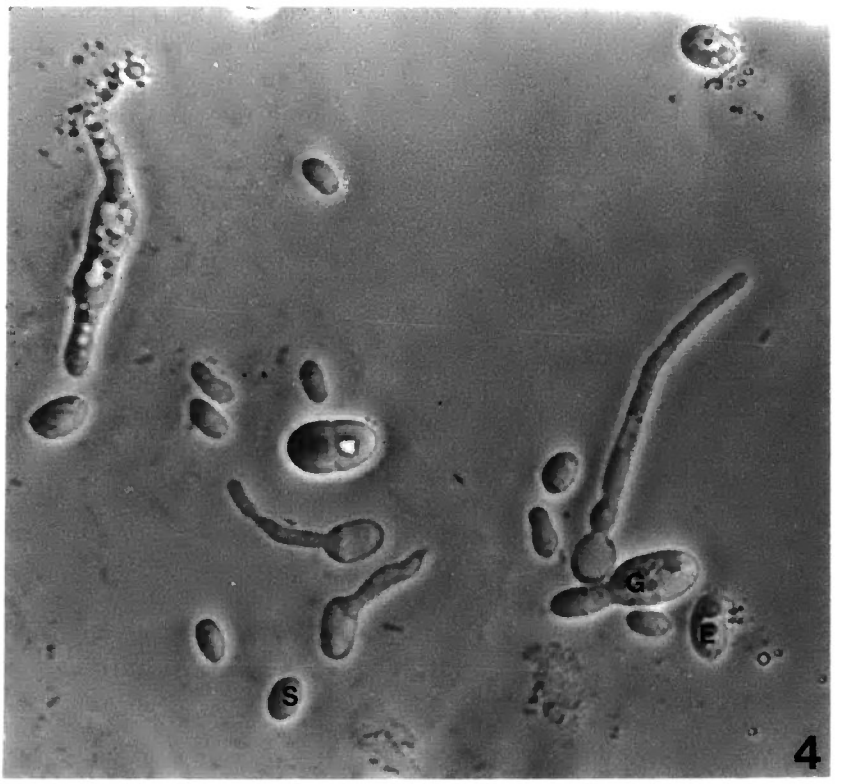
2



3

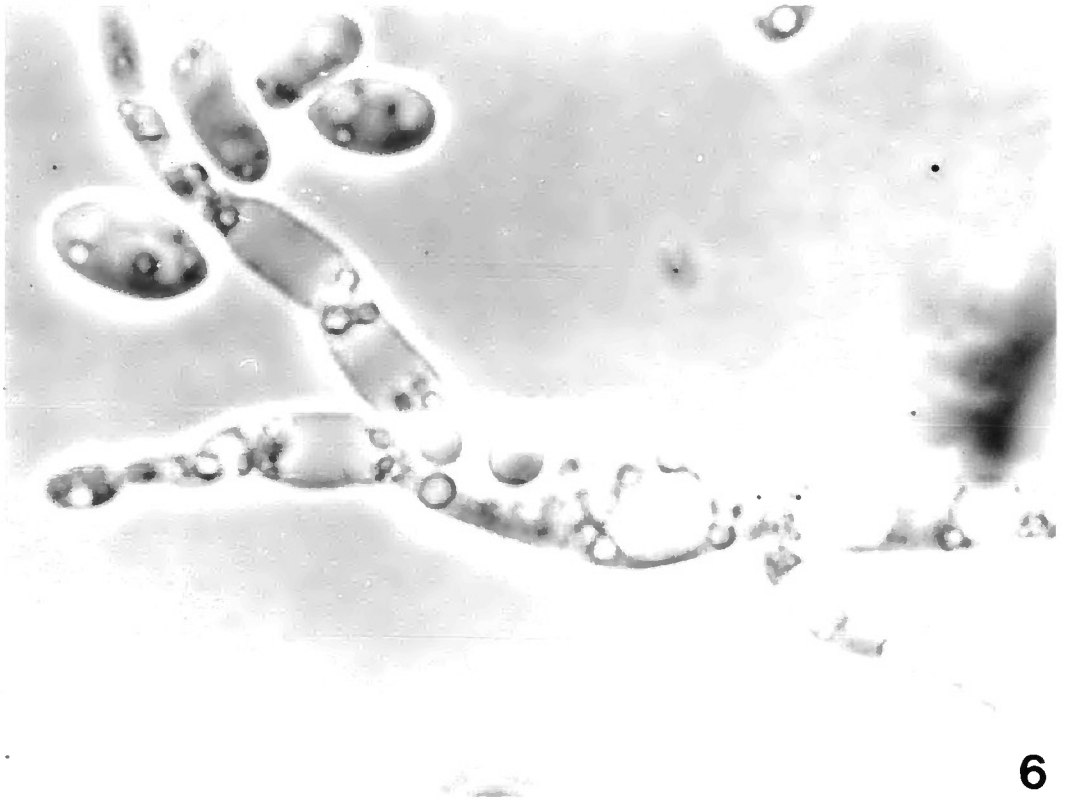
Fig. 4. Small (s), enlarged (E), and germinating conidia (G). X2,640.

Fig. 5. The appearance of a conidiophore (Co) producing a conidium (C). X6,160.


G
F
S
4


Co
C
5

Fig. 6. The appearance of a young MS, which occurs on third day. 6,160.



6

Conidia counts were made 3, 4, and 5 days after inoculation. The following data was tabulated in Table 2. On day 3, flask A contained 1.7×10^7 conidia/ml, flask B contained 3.1×10^7 conidia/ml, flask C contained 4.3×10^7 conidia/ml and flask D contained 4.5×10^7 conidia/ml. On day 4, the concentrations were 1.1×10^7, 2.7×10^7, 2.0×10^7, and 2.1×10^7 conidia/ml for flasks A, B, C, and D, respectively. On day 5, the concentrations were 1.45×10^7, 3.9×10^7, 3.5×10^7, and 4.0×10^7 conidia/ml for flasks A, B, C, and D, respectively. The results are shown graphically in Fig. 7. It appeared as though the higher the initial concentration of conidia, the smaller the clusters of MS formed. Conversely, the smaller the initial concentration of conidia, the larger the clusters of MS formed (Fig. 8).

Transmission Electron Microscopy

By means of electron microscopy, the three strains of V. dahliae were compared and contrasted at three days of growth in PGAM. The nucleus of t9 appeared irregular along its membrane (Fig. 9). In some cases the nucleoplasm appeared to extend out into the cytoplasm. There was apparent cytoplasmic organelle-nuclear continuity. The membraned elements of the apparently altered mitochondria appeared to extend into the nucleoplasm. Condensed chromatin material was scattered throughout the nucleoplasm. Endoplasmic reticulum (ER) was observed in close proximity of the nucleus. In some

Table 2. Production of conidia by different concentrations of conidia from day 3-5 in PGAM.

Days following inoculation	Number of conidia/ml of medium			
	A 1×10^3	B 1×10^4	C 1×10^5	D 1×10^6
3	1.7×10^7	3.1×10^7	4.3×10^7	4.5×10^7
4	1.1×10^7	2.7×10^7	2.0×10^7	2.1×10^7
5	1.45×10^7	3.9×10^7	3.5×10^7	4.0×10^7

Fig. 7. The effect of initial conidial concentration on MS synchronous development. The conidia concentrations were distinguished by letters (A...1 x 10^3, B...1 x 10^4, C...1 x 10^5 and D...1 x 10^6).

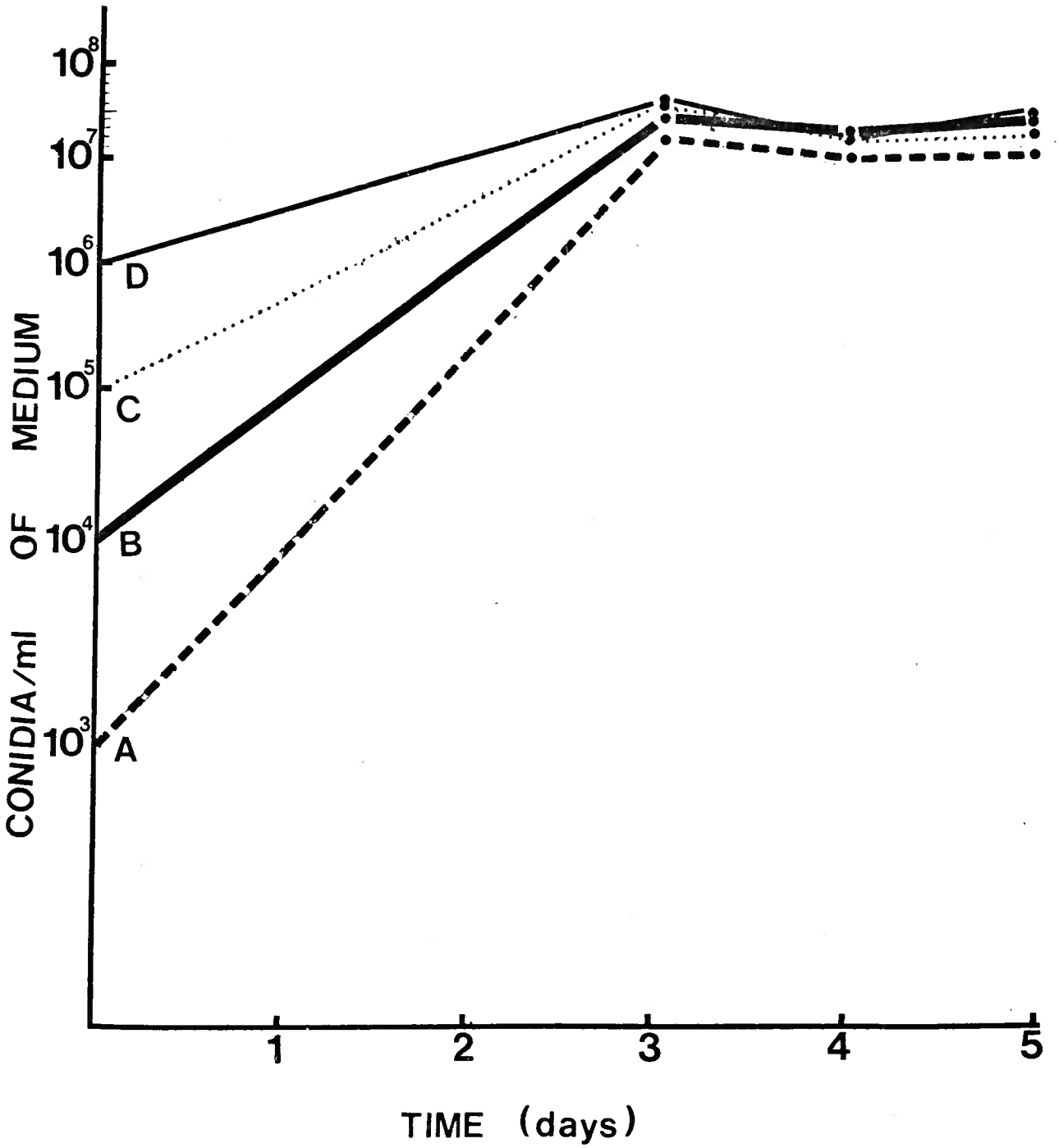
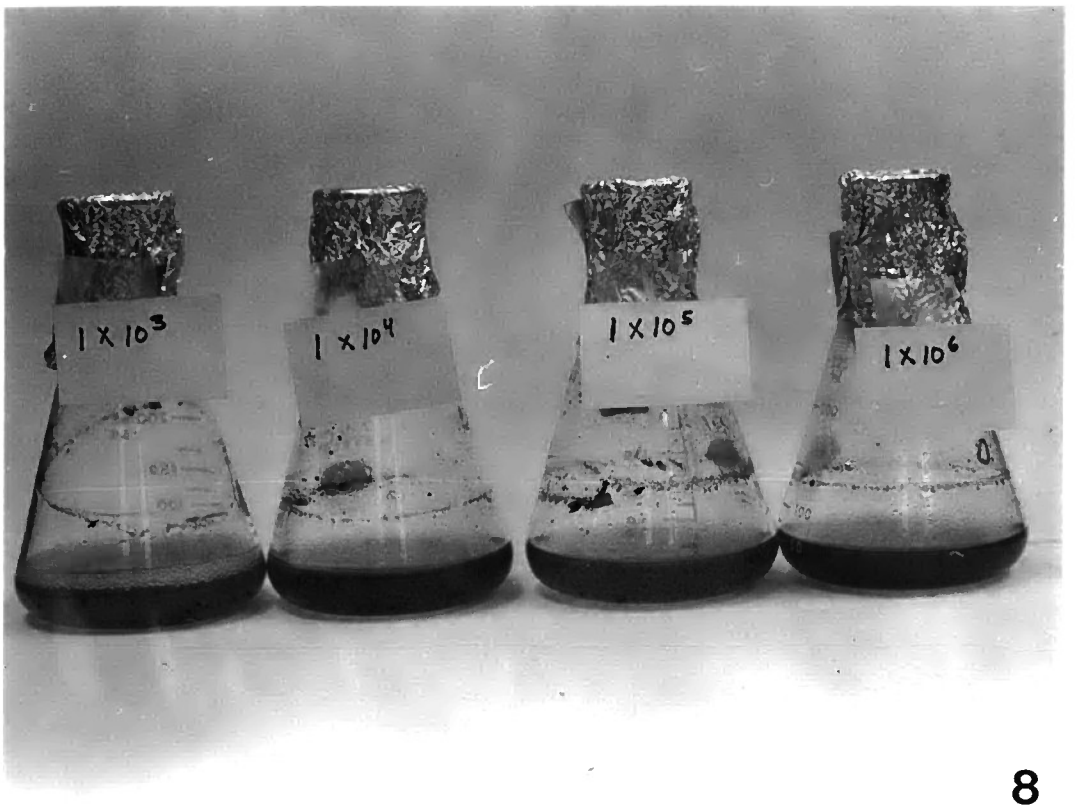

CONIDIA/ml OF MEDIUM
10^8
10^7
10^6
10^5
10^4
10^3
D
C
B
A
1
2
3
4
5
TIME (days)

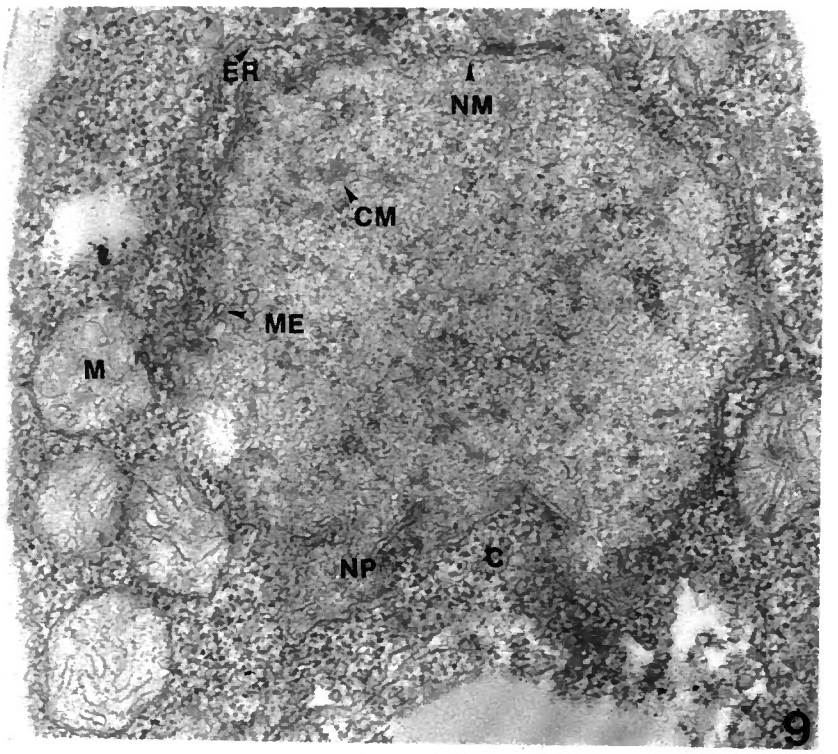
Fig. 8. Cultures of t9 illustrating the effect of conidial concentration on development of MS in PGAM.



8

Fig. 9. The wild-type (t9). Irregularities along the nuclear membrane (NM). Nucleoplasm (NP) extending into Cytoplasm (C). Membraned element ME) of a mitochondrium (M) extending into the nucleoplasm. Note appearance of endoplasmic reticulum (ER) and condensed chromatin material (CM). X62,900. Glutaraldehyde-Osmium tetroxide fixation (Ga-OsO_4).

Fig. 10. The wild-type. Nuclear membrane with apparent discontinuity. Note different shapes of mitochondria: circular (CU), eliptical (EL), and elongated (ED). X62,900. Ga-OsO_4.


ER
NM
CM
ME
M
NP
C
9

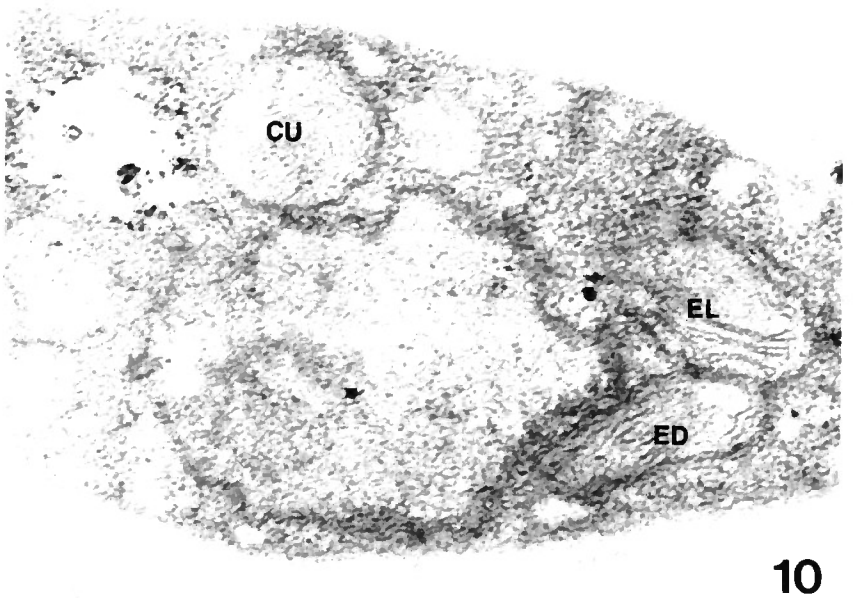
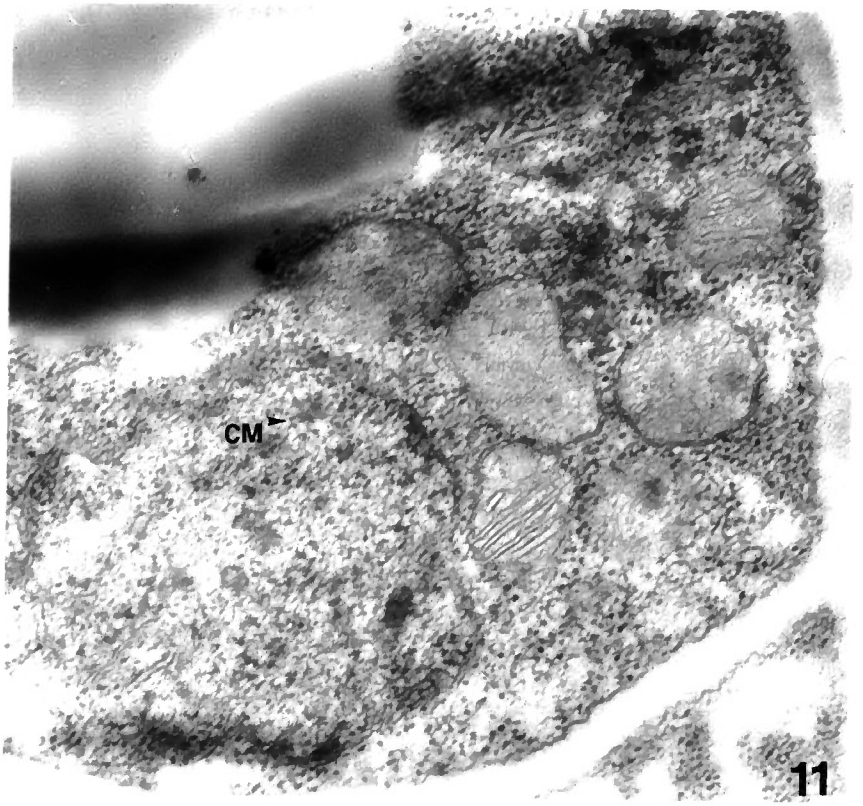

CU
EL
ED
10

Fig. 11. Albino 4. Appearance of very conspicuous chromatin material (Cm) along with symmetrical chromatin-associated structures. X62,900. Ga-OsO_4.

Fig. 12. Albino 4. Nucleus demonstrating symmetrical chromatin-associated structures (VLP). Continuity of ER with nuclear membrane (NM). Conspicuous chromatin material (Cm) and microtubule-like structures (MT) are found within the nucleus and cytoplasm. X62,900. Ga-OsO_4.


CM
11

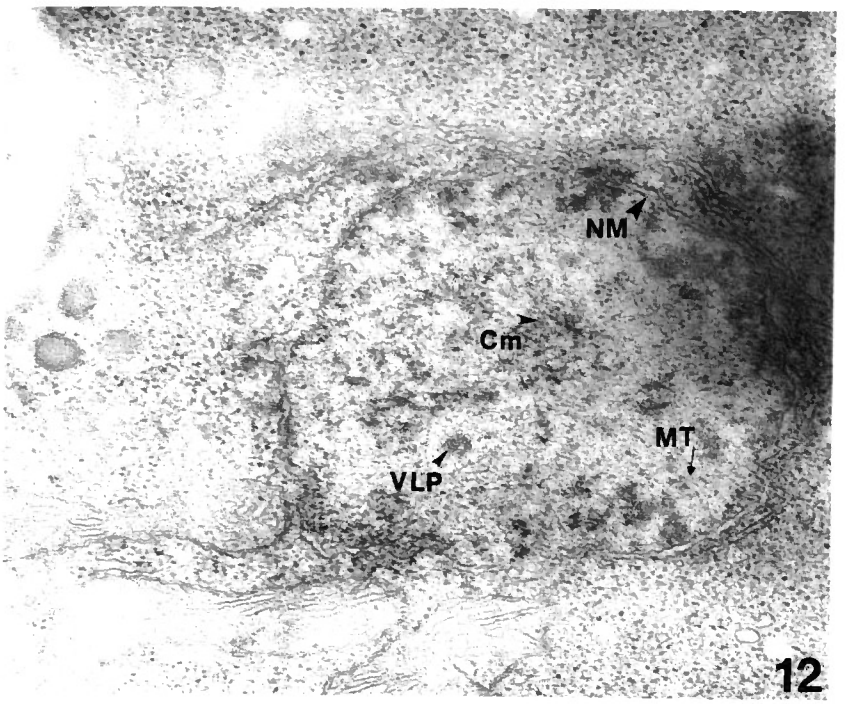
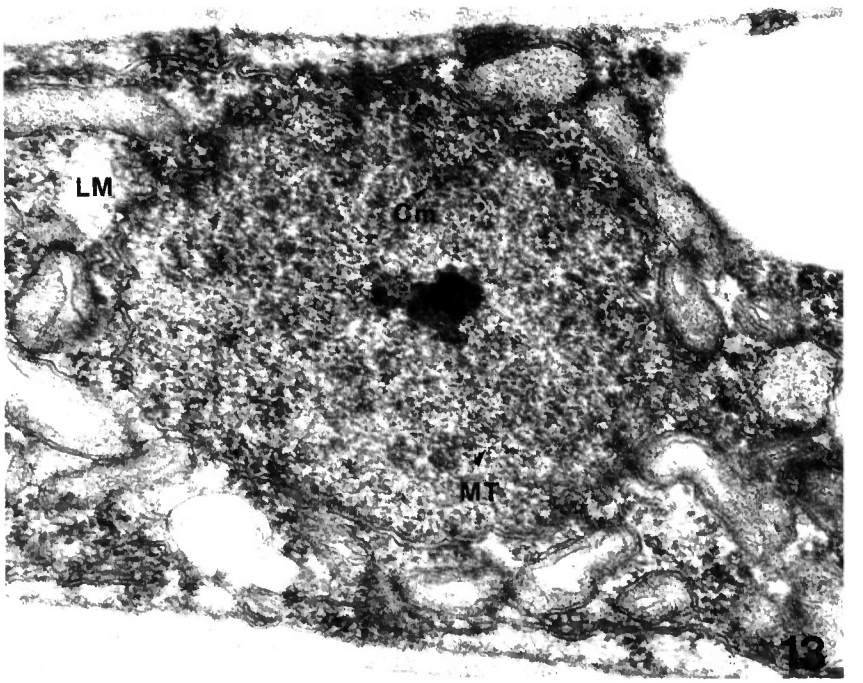

NM
Cm
MT
VLP
12

Fig. 13. Brown 1. Chromatin material (Cm) scattered within the nucleus. The cell exhibits lipid material (LM) and microtubule-like structure (MT) within the nucleus. X92,500. Ga-OsO_4.

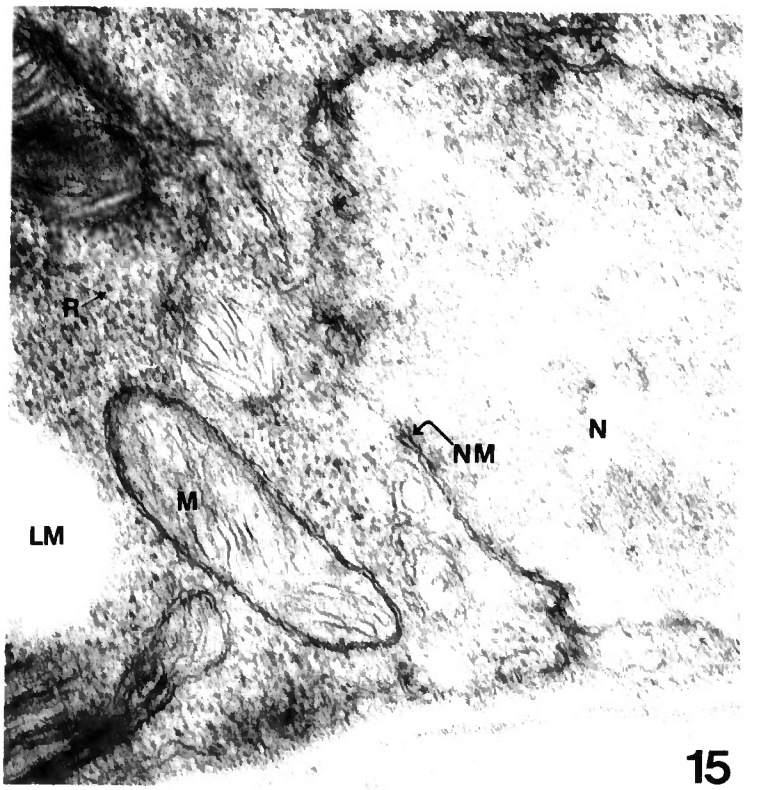
Fig. 14. Brown 1. Typical cell exhibiting double membrane-bound structures (DM). X92,500. Ga-OsO_4.


LM
Cm
MT
13


DM
14

Fig. 15. Brown 1. Nuclear membrane (NM) extending into cytoplasm. Note nucleus (N), lipid material (LM), mitochondria (M), and ribosomes (R). X129,500. Ga-OsO_4.

Fig. 16. Brown 1. Elongated mitochondria (ED) with circular one that has lost cristae (CU). X92,500. Ga-OsO_4.



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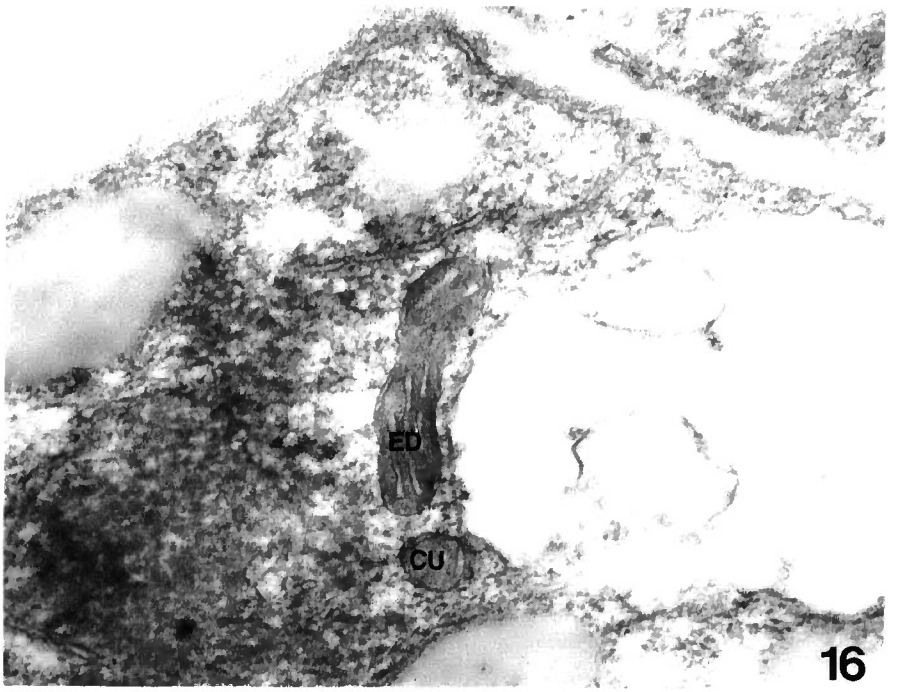


16

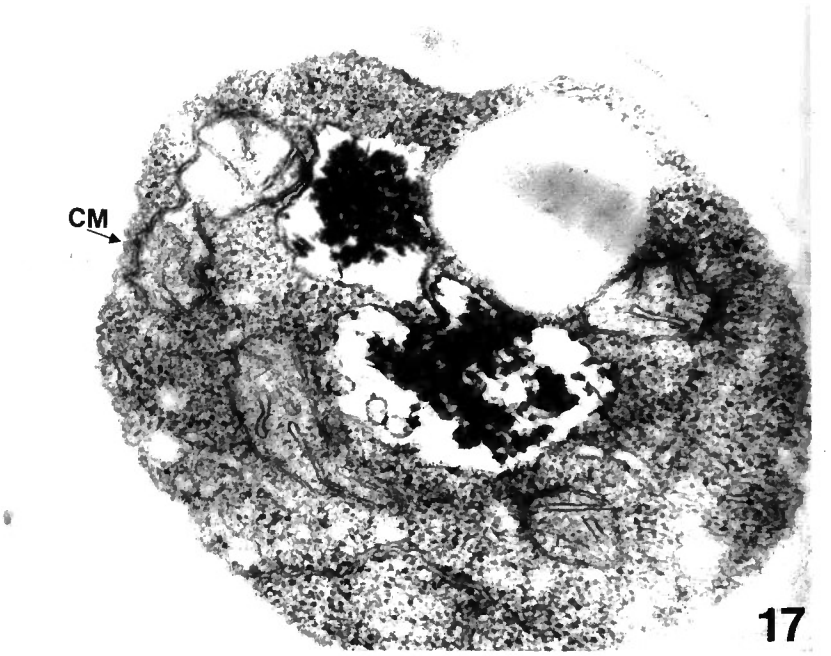
instances the nuclear membrane appeared discontinuous (Fig. 9) whereas in other instances it appeared to be continuous (Fig. 10). At certain points the ER appeared to be continuous with the nuclear membrane (Fig. 12).

The alm 4 nucleus seemed to be quite similar to that of t9, except for minor differences. For example, there was the appearance of very conspicuous chromatin material (Fig. 11 and 12) as well as in some cases, the appearance of symmetrical chromatin-associated structures within the nucleus (Fig. 11). These symmetrical chromatin-associated structures within the nucleus appeared quite regular (Fig. 11 and 12). They could be related to the virus-like particles (VLP) described by Hoch in his study of mycoparasitic relationships of Stephanoma parasitic on Fusarium (1978). In some cases there was a discontinuation of the nuclear membrane as observed in t9. Occasionally the ER of the cell was in close proximity of the nuclear membrane (Fig. 12) and in some instances seemed to be continuous with it. The nucleus of brm 1 appeared to be similar to the previous two to some degree in that there was the appearance of chromatin material within the nucleus (Fig. 13). The chromatin material was, however, more regular in distribution. In some cases the nuclear membrane seemed to be discontinuous and extended into the cytoplasm (Fig. 15).

The cell walls of the three were basically the same (alm 4, Fig. 19; brm 1, Fig. 20; t9, Fig. 21) with the

Fig. 17. Albino 4. Cell membrane (CM) showing double layer. X92,500. Ga-OsO_4.

Fig. 18. Brown 1. Electron-dense particles (EP) with filaments (F) extending out of them. X185,000. Ga-OsO_4.


CM
17

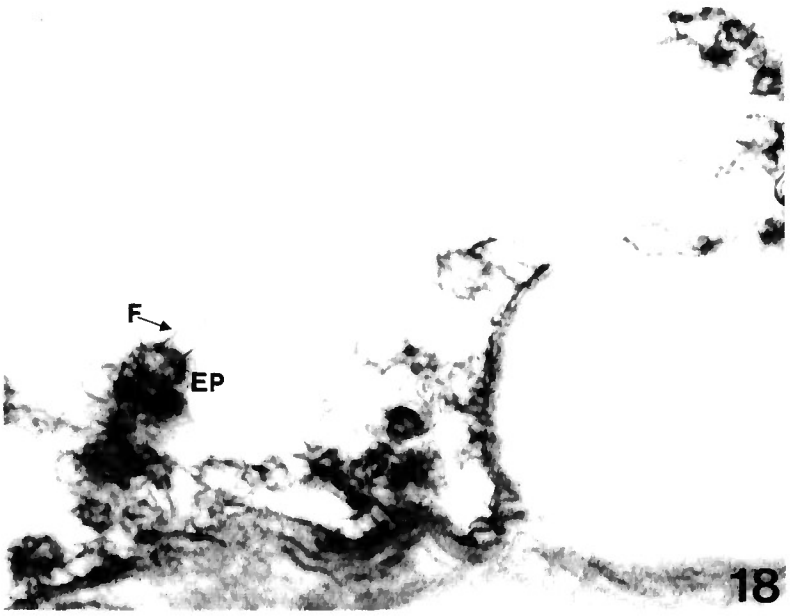
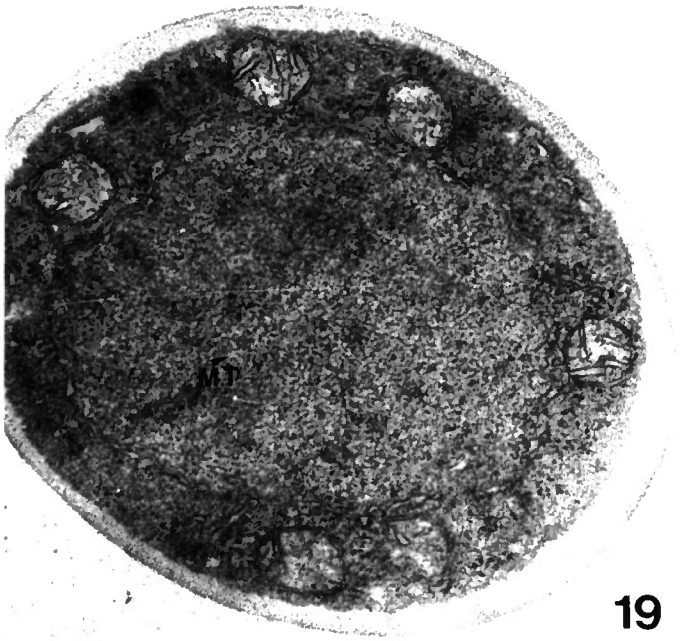

F
EP
18

Fig. 19. Albino 4. Cell with appearance of microtubule-like structure (MT) within the nucleus. X92,500. Ga-OsO_4.

Fig. 20. Brown 1. Cell exhibiting ER. Note the cell wall. X62,900. Ga-OsO_4.



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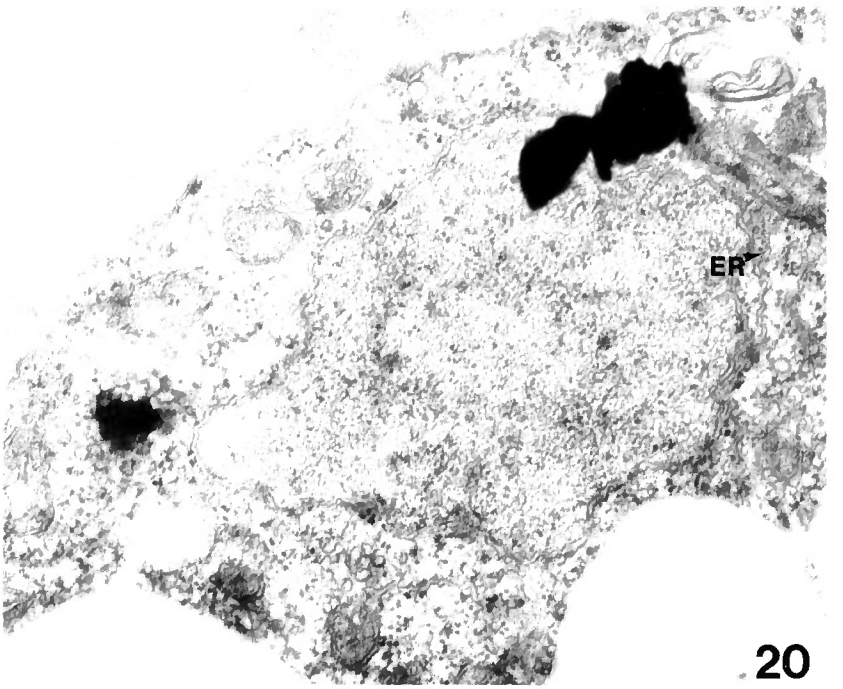

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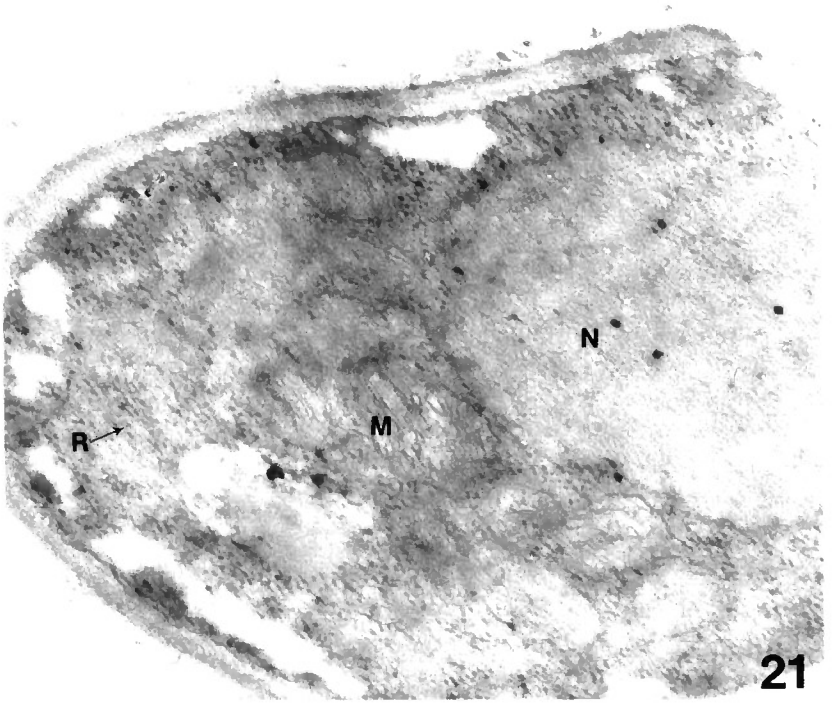
Fig. 21. The wild-type. Cell with typical organelles: mitochondria (M), nucleus (N), and ribosomes (R). Note the cell wall (CW). X44,400. Ga-OsO_4.

Fig. 22. Albino 4. Particles (P) embedded in cell wall (CW). Note the different shapes of mitochondria: circular (CU), elongated (ED), eliptical (EL). X90,000. Ga-OsO_4.



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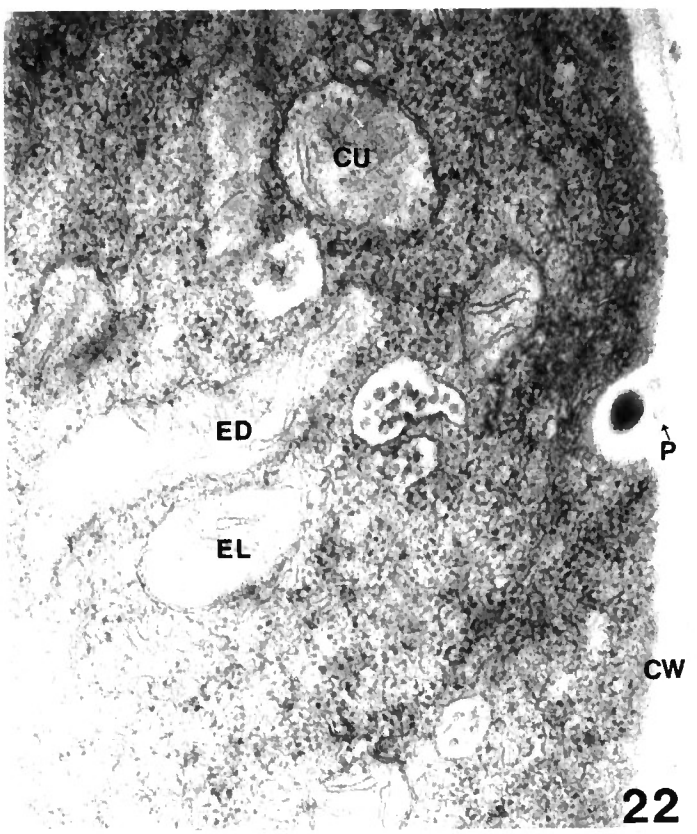
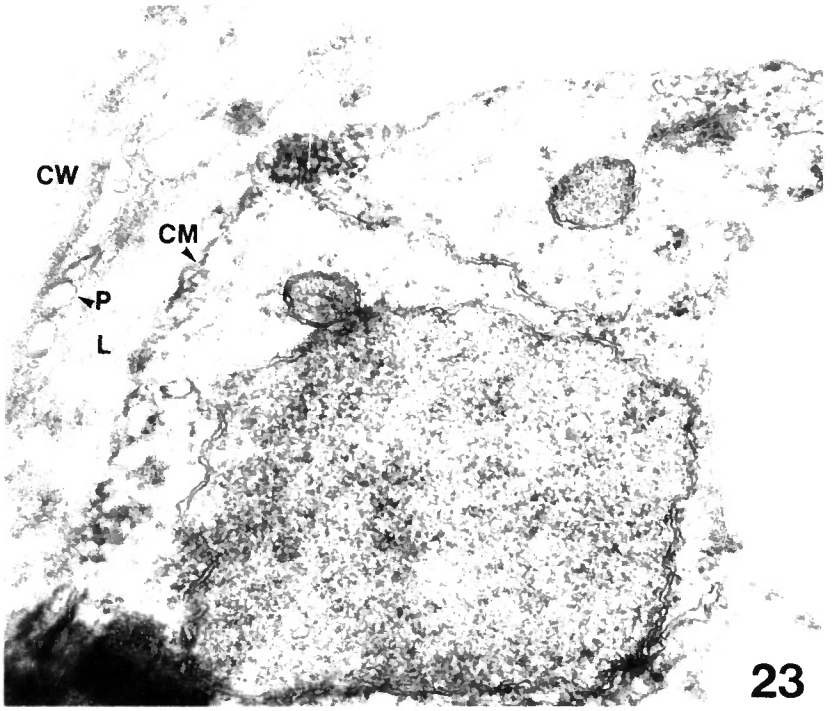


22

Fig. 23. Brown 1. Particles (P) embedded within cell wall (CW). Note invagination of cell membrane (CM) with intermediate layer (L) between cell wall and cell membrane. X92,500. Ga-OsO_4.

Fig. 24. The wild-type. Particles (P) embedded within cell wall (CW). Note invagination of cell membrane. X29,600. Ga-OsO_4.



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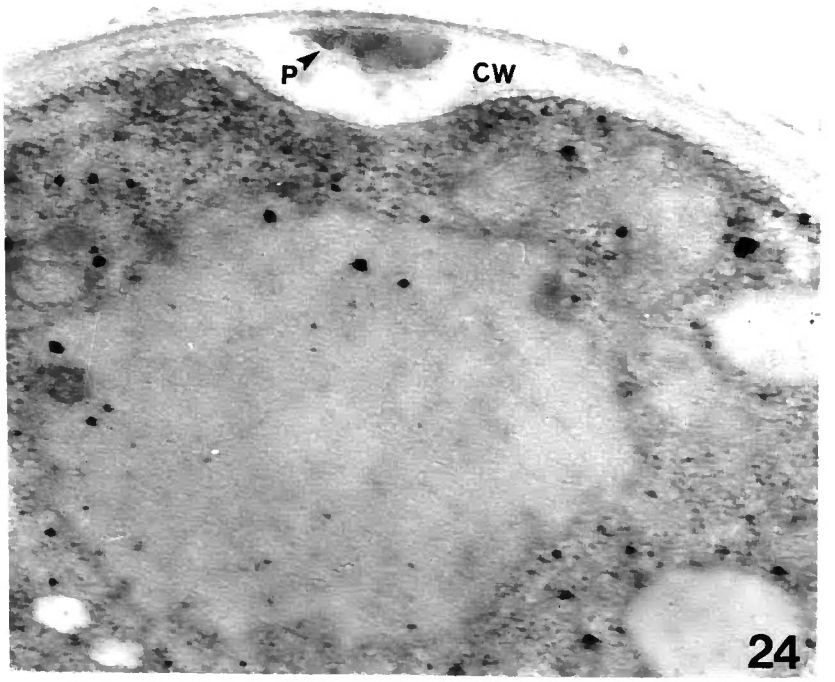


24

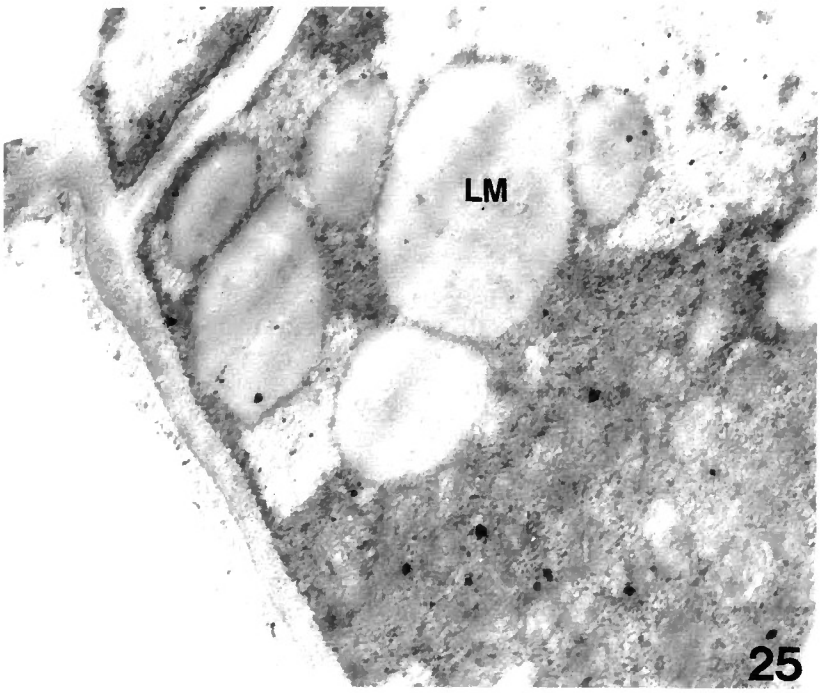
exception that in some cases there were particles embedded within the cell wall (alm 4, Fig. 22; brm 1, Fig. 23; t9, Fig. 24). In these cases, the cell membrane tended to be pushed inward. In Fig. 23, which is brm 1, there seemed to be some sort of layer between the cell wall and the cell membrane which contained particles or vesicles.

The cell membrane of t9 (Fig. 24, 25, and 26) appeared to be very similar to that of alm 4 (Fig. 17 and 27) and brm 1 (Fig. 13 and 20). In certain places you can see that it is a double membrane whereas in others you can not. The cytoplasms of the three were similar but they also tended to have distinguishing characteristics. Similarly, they consisted of nuclei, lipid material, mitochondria and ribosomes scattered throughout them (alm 4, Fig. 11; brm 1, Fig. 15; t9, Fig. 9). They differed in that t9 cytoplasm appeared more organized and less crowded when compared to alm 4 and brm 1. Alm 4 and brm 1 cytoplasm appeared more crowded by the presence of a greater amount of lipid material within them.

Lipid material was observed in all three strains. In t9 (Fig. 25 and 26) the lipid material tended to be scattered or localized in certain areas. In alm 4 and brm 1, the lipid material were scattered also (alm 4, Fig. 39; brm 1, Fig. 13) but at certain stages the lipid material seemed to be localized in particular areas (alm 4, Fig. 30; brm 1, Fig. 29 and 35).

Fig. 25. The wild-type cell demonstrating lipid material (LM). Note cell membrane. X29,600. $GaOsO_4$.

Fig. 26. The wild type cell demonstrating lipid material (LM). Note cell membrane. X62,900. $GaOsO_4$.


LM
25

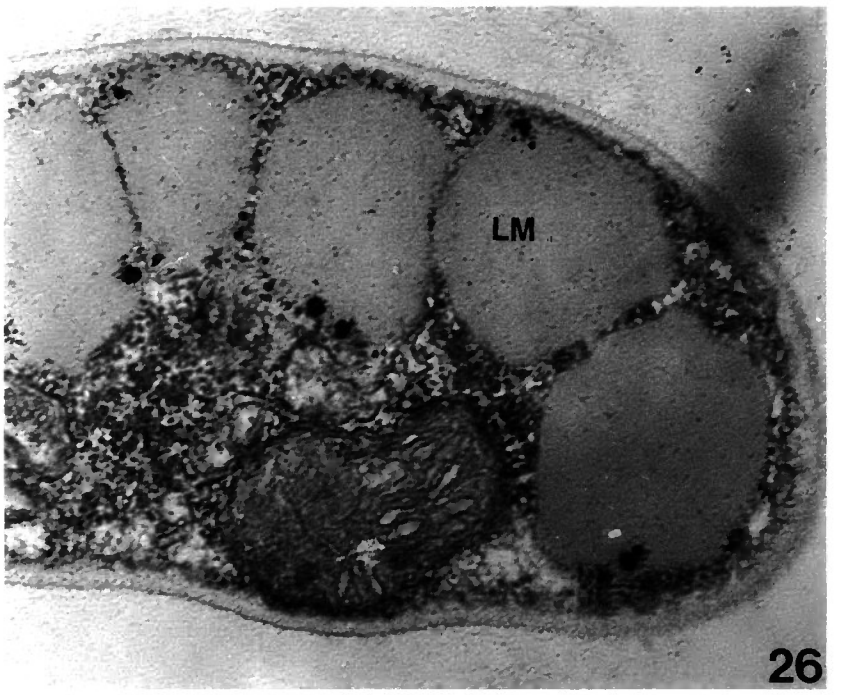
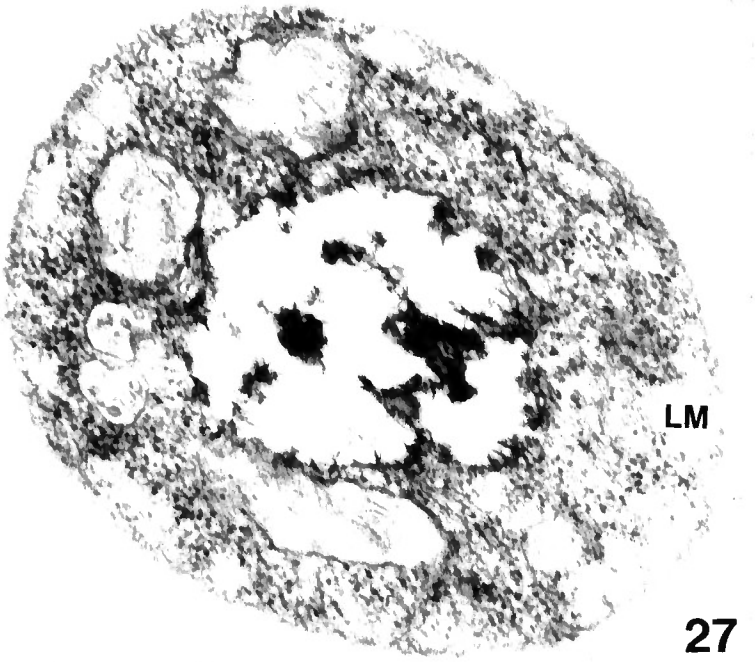

LM
26

Fig. 27. Albino 4. Cell exhibiting lipid material (LM). Note cell membrane. X62,900. Ga-OsO_4.

Fig. 28. Brown 1. Typical cell exhibiting double membrane-bound structures (DM). X92,500. Ga-OsO_4.


LM
27

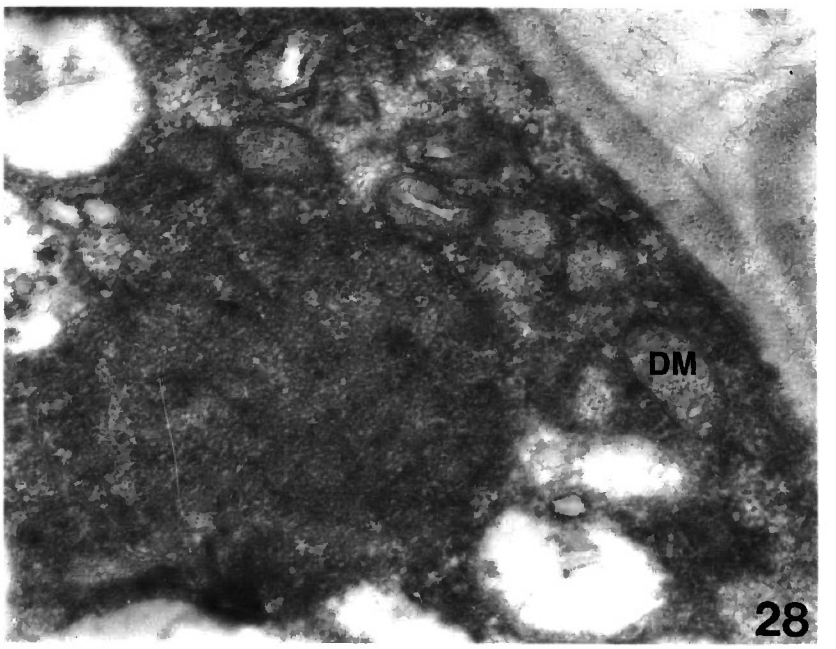
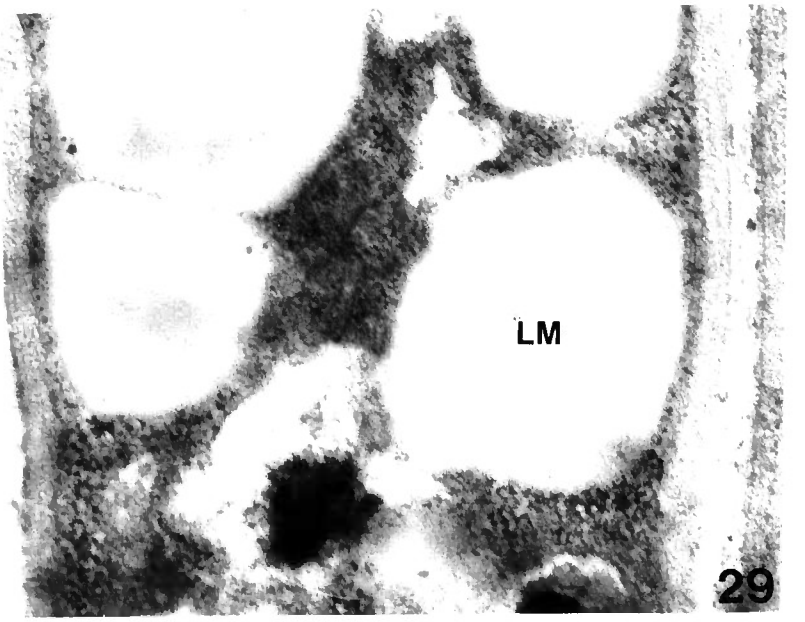

DM
28

Fig. 29. Brown 1. Cell showing the localization of lipid material (LM). X92,500. Ga-OsO_4.

Fig. 30. Albino 4. Cell showing the localization of lipid material (LM). X44,400. Ga-OsO_4.



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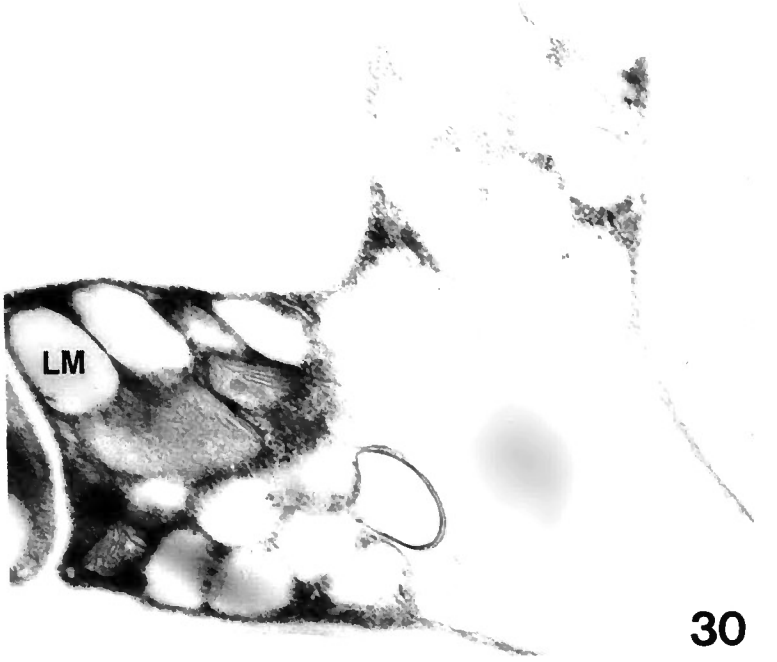
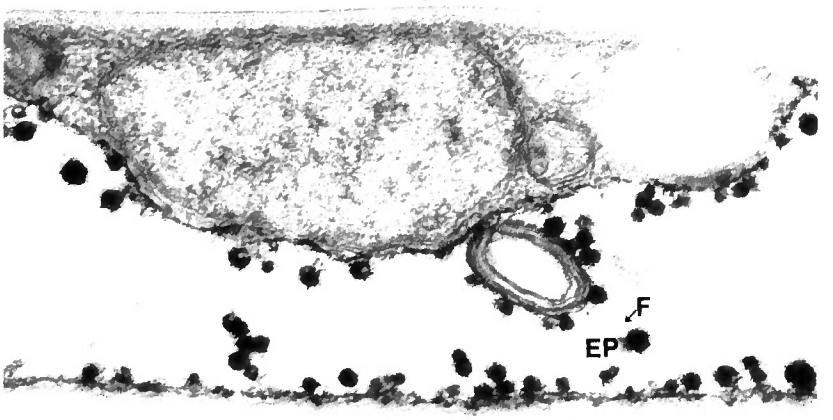


30

Fig. 31. Albino 4. Electron-dense particles (EP) with filaments (F) extending out of them. X92,500. Ga-OsO_4.

Fig. 32. Albino 4. Electron-dense particles (EP) with filaments (F) extending out of them. X92,500. Ga-OsO_4.



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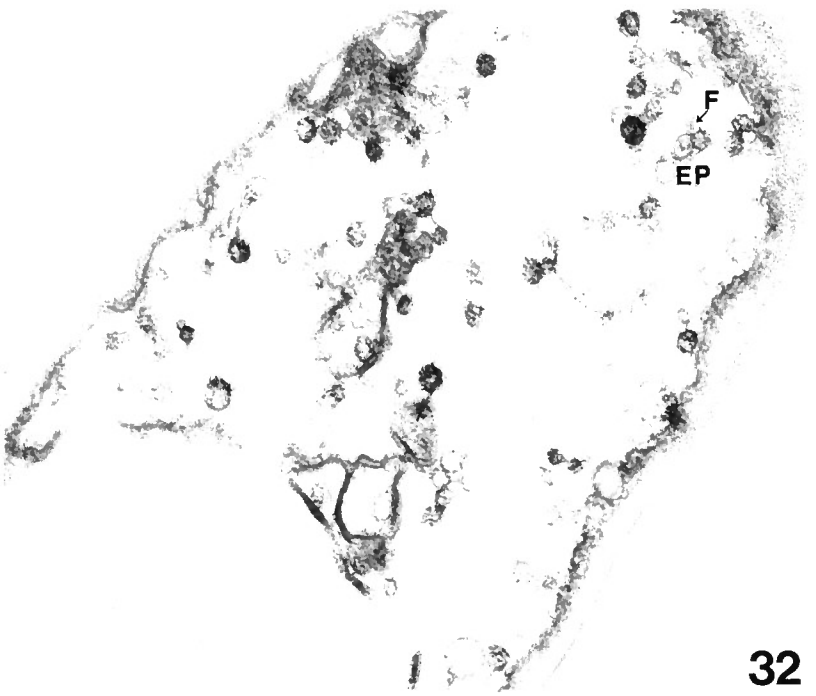
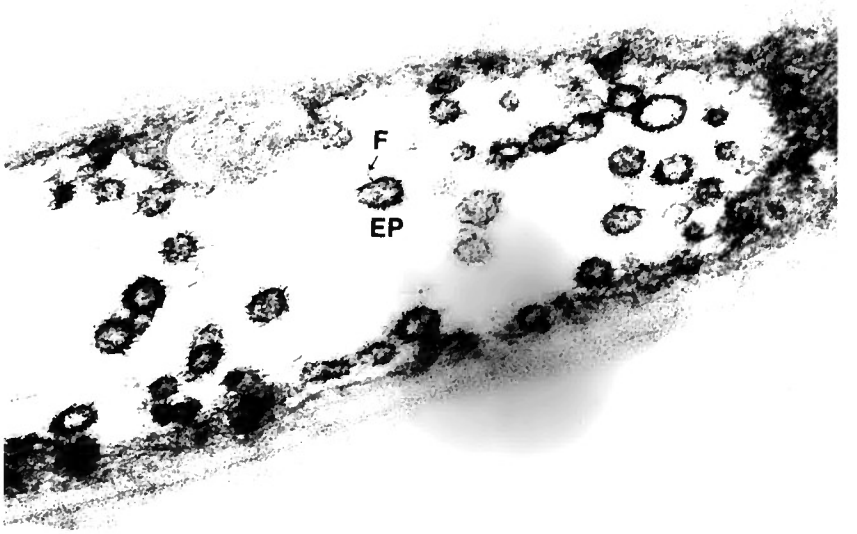


32

Fig. 33. Brown 1. Electron-dense particles (EP) with filaments (F) extending out of them. X129,500. Ga-OsO_4.

Fig. 34. Brown 1. Electron-dense particles (EP) with filaments (F) extending out of them. X185,000. Ga-OsO_4.



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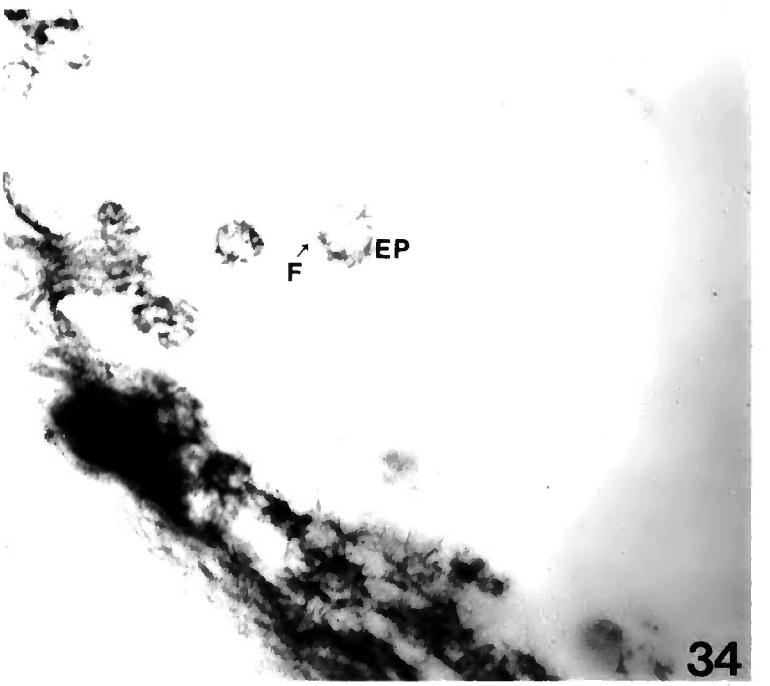
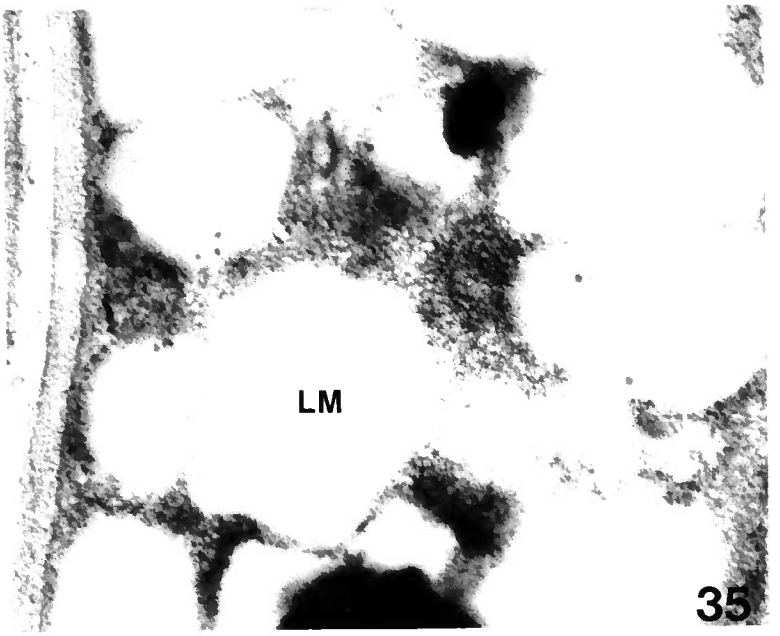


34

Fig. 35. Brown 1. Localization of lipid material (LM) within the cell. X44,400. Ga-OsO_4.

Fig. 36. The wild-type. Microtubule-like structures (MT) within the cytoplasm. Note the shapes of mitochondria: circular (CU) and elongated (ED). The elongated mitochondrium has some sort of body embedded in it. X62,900. Ga-OsO_4.



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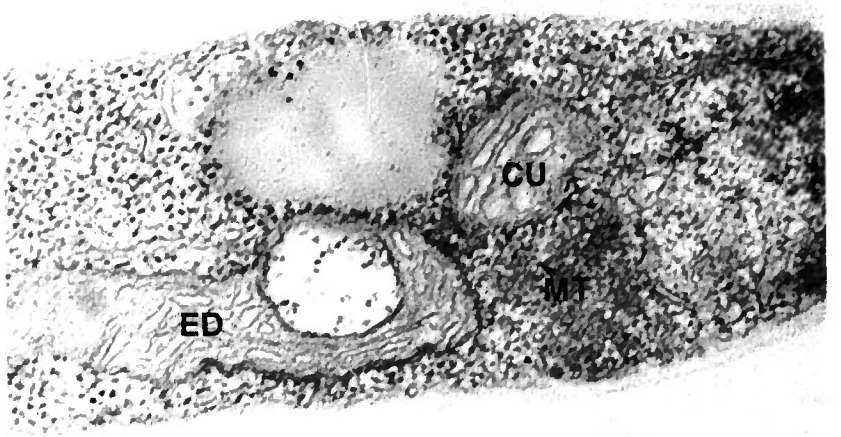


36

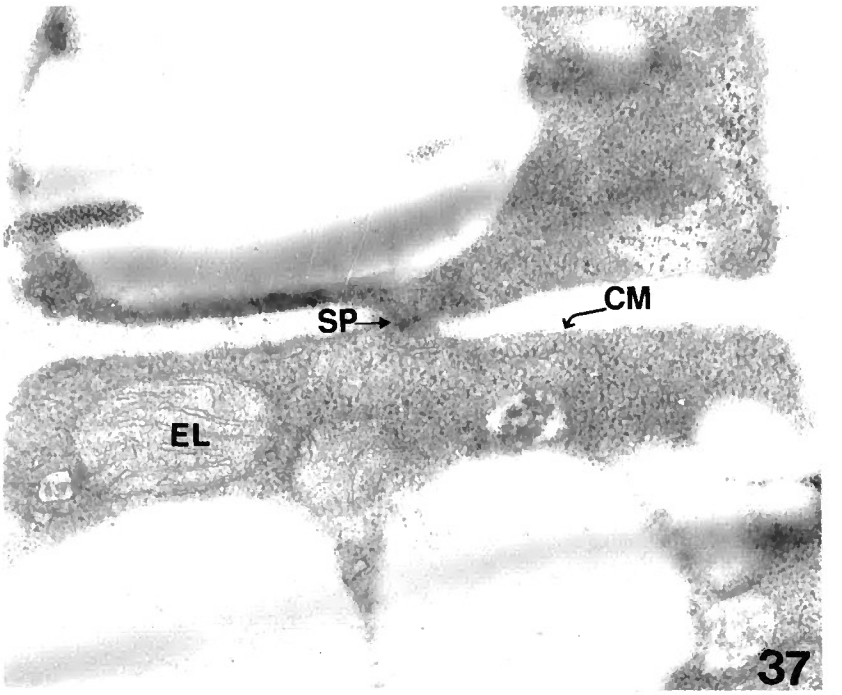
In some cases microtubule - like structures were seen in the nucleus and cytoplasm of all three strains. The alm 4 nucleus (Fig. 12 and 19) displayed these structures while alm 4 cytoplasm (Fig.12) showed the same structures. The nucleus of brm 1 (Fig. 13) showed these structures while brm 1 cytoplasm (Fig. 40) also showed similar structures. The nucleus of t9 (Fig. 10) as well as cytoplasm (Fig. 36) also showed these structures.

There was a peculiar thing about alm 4 and brm 1. Within certain cells, there was an abundance of particles with filaments running out of them (alm 4, Fig. 31 and 32; brm 1, Fig. 18, 33 and 34). These particles were never observed in t9. In brm 1 they tended to show up more often than in alm 4.

The mitochondria of all three strains showed different alterations. Mitochondria ranged from circular to elongated in shape. These different changes were shown by the following figures (t9, Fig. 10 and 36; alm 4, Fig. 11, 22, 37 and 38; brm 1, Fig. 16 and 40). The most peculiar thing was that in alm 4 and t9 there was an appearance of single membrane-bound structures that coincided with the disappearance of cristae in mitochondria (alm 4, Fig. 43; t9, Fig. 42). It is believed that the mitochondria are converted to these structures, which are labeled peroxisomal-like structures. These structures were not found in brm 1. On the other hand brm 1 had many double membrane-bound

Fig. 37. Albino 4. Note the cell membrane (CM) and eliptical mitochondria (EL). A strand of ER is in front of the septal pore (SP). X90,000. Ga-OsO_4.

Fig. 38. Albino 4. Note the different shapes of mitochondria: elongated (ED), eliptical (EL) and one dividing (DM). X92,500. Ga-OsO_4.


SP
CM
EL
37

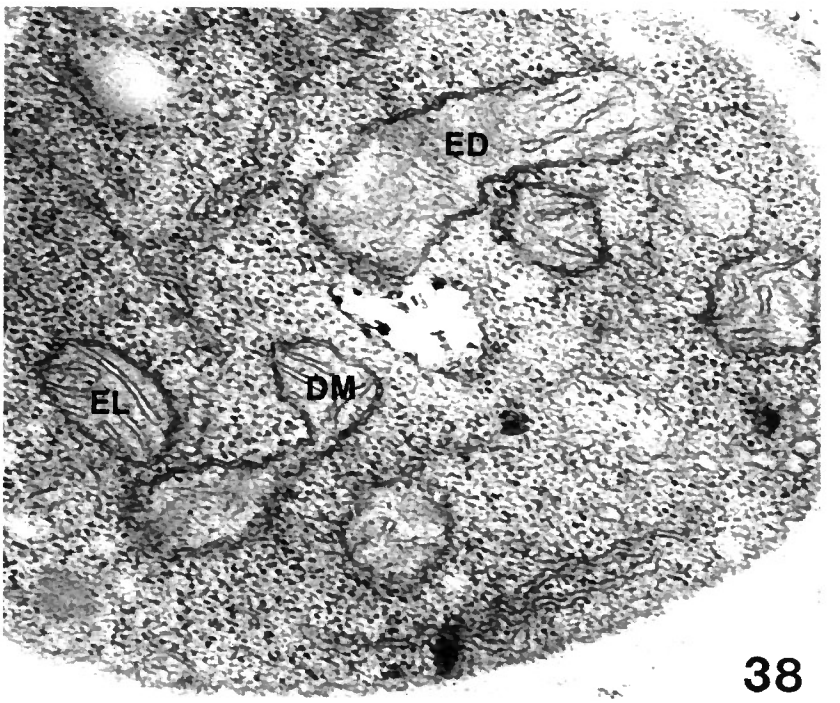

ED
EL
DM
38

Fig. 39. Albino 4. Note the appearance of lipid material (LM). X90,000. Ga-OsO_4.

Fig. 40. Brown 1. Note the microtubule-like structures (MT) within the cytoplasm. Note the shapes of mitochondria. X129,500. Ga-OsO_4.

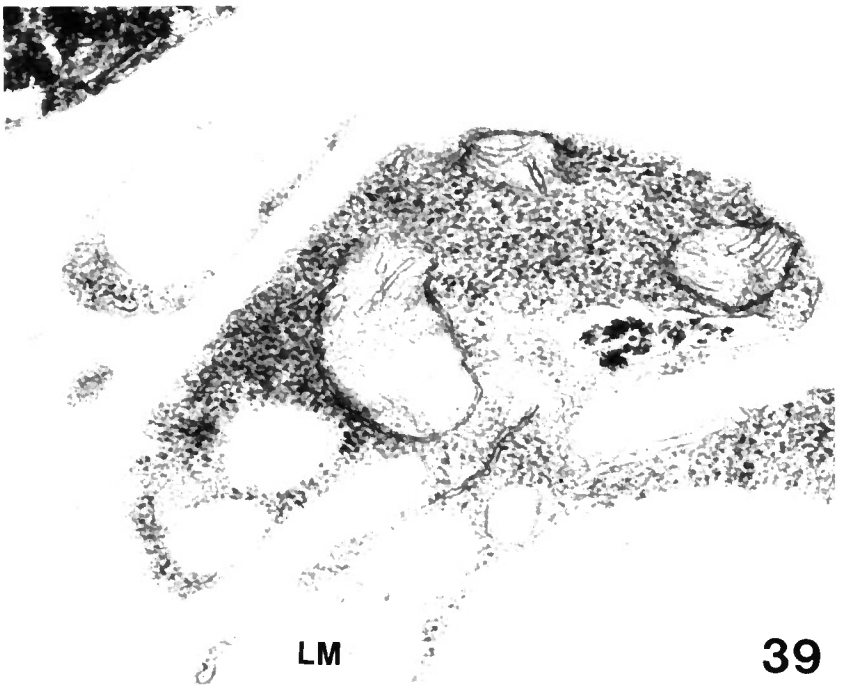


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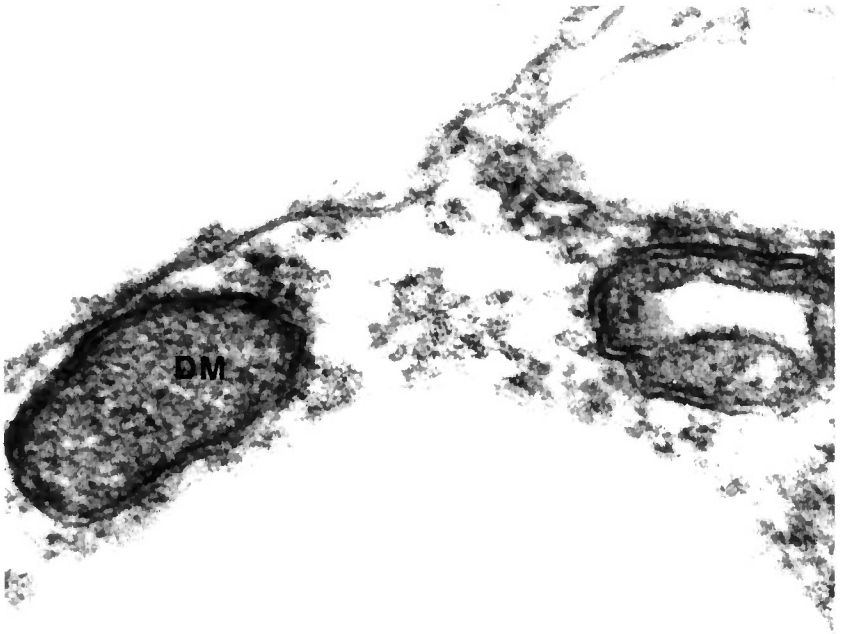
Fig. 41. Brown 1. Showing double membrane-bound structures (DM). X185,000. Ga-OsO_4.

Fig. 42. The wild-type. Exhibiting single membrane-bound structure (P). X92,500. Ga-OsO_4.



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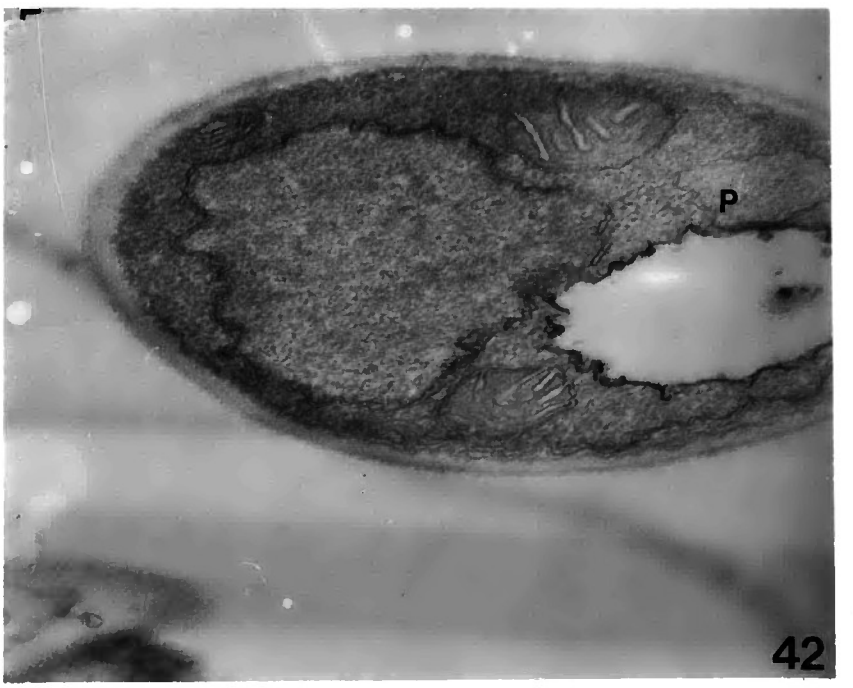

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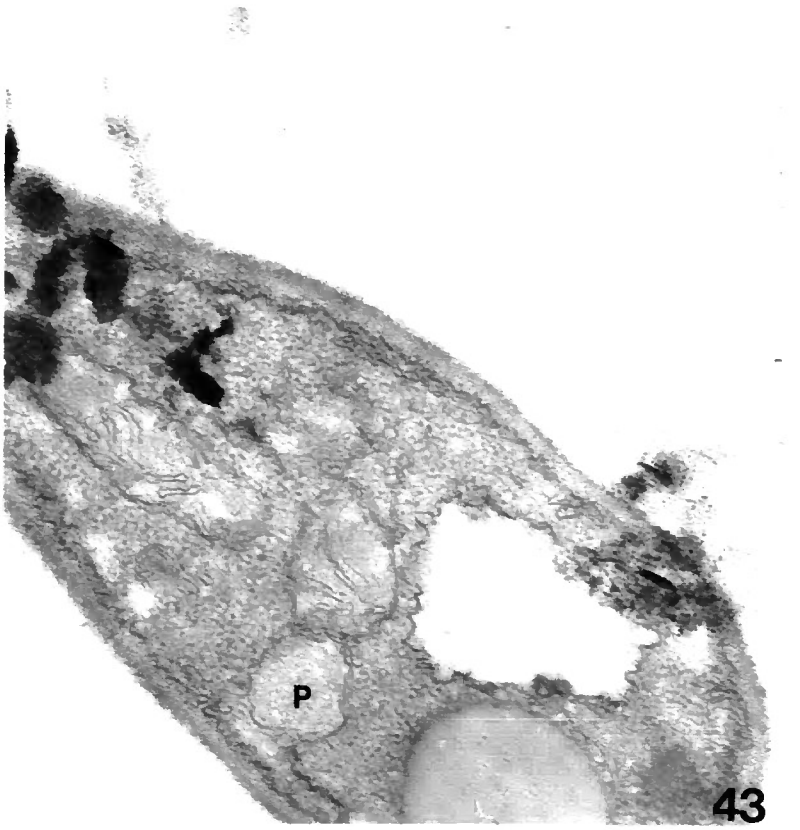
Fig. 43. Albino 4. Showing single membrane-bound structure (P). X92,500. Ga-OsO_4.


P
43

structures which resembled mitochondria that have lost their cristae (Fig. 14, 28 and 41). It is believed that brm 1's mitochondria have the ability to lose their cristae but they do not have the ability to convert their double membranes into single membranes. It is also believed that these single membrane-bound structures are very vital in the pathway of melanin synthesis. It has been shown that alm 4, which produces no pigment, can be grown in the presence of brm 1, which produces a brown pigment and the results will be the production of the normal black pigment produced by t9 (Bell et al., 1976).

CHAPTER V

DISCUSSION

The mode of conidiation, leading to MS development was basically identical for all three strains except for the deviation in alm 4 which resulted in a gradual, constant increase in conidia after two days in PGAM as opposed to the leveling off of conidial production at the same point for t9 and brm 1. The basic pattern of conidial production in all three strains is similar to the process described by Taylor (1978) for t9. She suggested that there was a rapid proliferation of conidia during the first 24 hr by auto-conidiation. Auto-conidiation has previously been observed in V. dahliae (Tolmsoff, personal communication). Taylor (1978) further suggested that the conidia were necessary for the production of MS.

The similarity of conidiation patterns among the three strains indicates that MS development proceeded by basically identical patterns. The variation observed in alm 4 after 2 days was of interest since there was neither melanin nor any of its pigmented intermediates produced. Fungal conidiation has been reported to be regulated by a number of chemical and physical factors (Smith, 1978). The fact that conidial proliferation ceases in the pigmented MS strains earlier than in albino strains suggests that melanin

or its intermediates might affect the conidiation process as related to MS development.

The data obtained from experiments designed to determine the most effective conidial concentration for the inoculation of synchronous cultures suggest that there were factors other than inoculum concentration limiting the maximum number of conidia produced in PGAM. Although one of the conidial concentrations (1×10^6) was observed to produce the highest degree of synchrony, no more conidia were produced at the end of five days than those cultures inoculated with fewer conidia. It has been reported that sclerotia development may be induced in other fungi by accumulated metabolites in the medium (Willetts, 1978). It may be possible that the substances which accumulate in PGAM may serve to inhibit conidial production and initiate the development of MS. The possibility of a crowding effect due to competition for substances in the medium is not ruled out.

Some of the more obvious differences among the three strains were at the ultrastructural level. According to previous studies the third day represents the point at which young MS begin to appear in the medium (Mayfield and Taylor, 1978; Taylor, 1978).

The irregularity of the nuclear membrane in t9 was also reported by Taylor (1978) as well as the extension of the nucleoplasm into cytoplasmic regions. In the present

study the discontinuity of the nuclear membrane was more apparent than reported by Taylor. This could be a means by which genetic alterations come about in MS, since it has been suggested that MS are the source of genetic variability (Tolmsoff, 1972). The observation of condensed chromatin material that was scattered throughout the nucleoplasm could be considered a difference between t9 and both mutants.

The chromatin material in alm 4 appeared more intensely stained than that of t9 while brm 1 chromatin material appeared more dispersed. These differences could represent different degrees of mitotic activity. The intensely stained chromatin material has been associated with rapidly proliferating cells in E. coli (Paul, 1972). This could possibly be the case in V. dahliae also. Thus, this condition in alm 4 could correlate with the continued increase in conidia after 3 days. The symmetrical chromatin-associated structures were similar to VLP described by Hoch (1978). Viruses have been reported to occur in fungi (Hollings and Olwen, 1969). The observation of these VLP suggest that further studies should be done since the ultrastructure of alm 4 has not been previously described.

Basically, the cell membrane of all three were similar sometimes having particles embedded within the cell wall. The accumulation of small vesicles was unique to brm 1 and of interest in that brm 1 secretes (+)-scytalone into the medium (Bell et al., 1976). Griffiths (1970) demonstrated

that these were real structures and not artifacts in V. dahliae. Vesicles or paramural bodies have been suggested to transport substances out to the cell wall to participate in cell wall metabolic activities (Mayfield, 1974). Although no melanin or melanin intermediates have been identified within cytoplasm, Wheeler et al. (1976) have reported the deposition of melanin intermediates at the surface of the cell. These vesicles could be transporting (+)-scytalone out to the cell wall.

One of the most noteworthy differences found among the three strains was the occurrence of single membrane-bound structures in alm 4 and t9 whereas they were absent in brm 1. On the other hand, a larger number of double membrane-bound structures was prevalent in brm 1. It is believed that these structures whether in alm 4, brm 1, or t9 developed from mitochondria. Stages of mitochondria alterations leading to the development of these membrane-bound structures were described by Mayfield and Taylor (1978). These single membrane-bound bodies were reported to appear in MS prior to melanin deposition. Bell et al. (1976) demonstrated that alm 4 had some presumable enzyme system which could convert the (+)-scytalone of brm 1 into melanin. As suggested by Bell et al. (1976), the genetic alterations in the mutants are in different locations. It is suggested that the ones occurring in alm 4, occurred much earlier along the chromosome than the one in brm 1, which could account for alm

4 inability to produce the precursor, but on the other hand, allowed it to use the produced precursor to synthesize melanin. The single membrane-bound structures are found in t9 and t9 produces melanin, while these structures are also found in alm 4, it does not produce melanin. However, alm 4 is able to convert (+)-scytalone to melanin, suggesting that the same enzyme system is functioning in alm 4 and t9. Since peroxidase enzymes have been demonstrated to occur in both pigmented and hyaline strains of Verticillium (Gafoor and Heale, 1971), it is suggested that these single membrane-bound structures might be peroxidase-transporting structures. Data from this investigation suggest that brm 1 is lacking the enzyme system, which might be localized in the single membrane-bound structures of alm 4 and t9.

CHAPTER VI

SUMMARY

The development of microsclerotia in wild-type and microsclerotial mutants of V. dahliae, growing in polygalacturonic acid medium (PGAM), was studied by a combination of phase contrast and electron microscopic techniques. Samples were taken from the medium at 24 hr intervals up to five days and prepared for phase or electron microscopic observations. The modes of conidiation, leading to MS development in the three strains, appeared to be the same. The albino strain did seem to differ, in that it had a continuous increase in conidiation from 2-4 days while the brown and wild-type strains showed only a gradual increase. The use of various concentrations of conidia for inoculum suggested that there were factors other than inoculum concentration which limited the maximum number of conidia produced in PGAM. At the electron microscopic level, the chromatin material of the three appeared different. The most noted difference among the three was the appearance of single membrane-bound structures in the albino and wild-type strains whereas the brown strain did not have these.

The overall investigation suggests that the wild-type and albino strains of V. dahliae have an enzyme system which is missing in the brown strain. This melanin mediating system

appears to be the peroxisomal-like structures, which are missing in the brown strain.

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